BIOCHIP JOURNAL, Vol. 3, No. 2, 113-118, June 2009

Characterization of Monoclonal Antibodies against Adenylate Kinase Isozyme 3: a New Cardiac Disease Diagnostic Marker

Yong Goo Yoo[1], **Hee Jong Lee**[1]
& Hyo Joon Kim[1]

[1]Department of Biochemistry, Hanyang university,
Sa-Dong, Sangrok-Gu, Ansan, Gyeonggi-Do, Korea
Correspondence and requests for materials should be addressed
to H.J. Kim (kimhj104@hanyang.ac.kr)

Accepted 8 May 2009

Abstract

Human mitochondrial adenylate kinase plays an important role in energy metabolism by phosphoryl transfer between adenine nucleotides. Our previous studies had strongly suggested that the serum levels of human adenylate kinase 3 (hAK3) should be a biochemical marker for the necrosis of myocytes. Also, it was reported that the expression of AK3 was induced about 3 fold in the condition of hypoxia. Acute myocardial infarction (AMI) is a disease appeared when cardiac muscle cell disrupted by hypoxia. Monoclonal antibodies (Mabs) are produced widely for use in researches and also are produced in large quantities against a plethora of antigens for use in diagnosis and sometimes in treatment. In this study, B lymphocyte hybridoma cell lines producing MAb against hAK3 were constructed and characterized. For the proper and optimal applications, antibodies were grouped and characterized according to their physicochemical properties and antigenic structural characters. Identification and compairson for the complementarity determining regions (CDRs), the avidities in terms of dissociation constants and isotypes were made for each Mabs. This Mabs are useful in protein diagnostic lab-on-a-chip technology.

Keywords: Adenylate kinase, Monoclonal antibody, AMI, Diagnostic marker

Introduction

Adenylate kinase (AK) catalyses a phosphoryl transfer reactions between adenine nucleotides and is found ubiquitously in organisms. The enzyme plays important roles in homeostatics of adenine nucleotides, signal transduction, and in energy metabolism[1-3].

AK forms an isozymes (AK1-AK6) have so far been characterized in vertebrates[1,4-6].

AK3 is a mitochondrial matrix specific isozyme of AK family which utilizes GTP instead of ATP as phosphate donor.

In the previous study, it had been demonstrated that AK3 was expressed in heart, liver and kidney, but not in any other tissues including skeletal muscle[7-9]. For this reason, AK3 was strongly suggested as a biochemical marker for the diagnosis of acute myocardial infarction (AMI).

AMI is a disease appeared when cardiac muscle cell disrupted by hypoxia. Presently myoglobin, troponin T (TnT), troponin I (TnI), and creatine kinase-MB isozyme (CK-MB) are used as diagnostic markers for the AMI. Also, it was reported that AK3 gene was induced in the condition of hypoxia[10,11]. Thus the measurement of AK3 levels in serum was elevated in patients with early stage of AMI.

Monoclonal antibodies (MAbs) are produced widely for use in researches and also are produced in large quantities against a plethora of antigens for use in diagnosis and sometimes in treatment. We had development of MAbs against AK3 and characterized the MAbs obtained.

We suggest that this MAbs can be apply to the diagnostic lab-on-a-chip technology.

Results and Discussion

Production of Antibodies

Nine MAbs specific for human mitochondrial acenylate kinase 3 were established.

The MAbs of the present invention are different from the other antibodies against conventional biochemical markers for the diagnosis of myocardial infarction, the MB isoenzyme of creatine kinase (CK-MB), troponin T and troponin I, in terms of having a very weak cross-reactivity to the known adenylate kinase isozyme 1 and 2. ELISA was used to determine the cross-recognition capabilities the monoclonal antibodies of SJB3-31 through 39 have for AK1, AK2 and AK3. We found that all monoclonal antibodies showed a specific response to AK3 only, without having any cross-recognition capability for AK isozymes (except AK3) (Table 1).

Table 1. Cross-recognition of AK isozymes by monoclonal antibodies.

	AK1	AK2	AK3
SJB3-31	0.056	0.042	1.059
SJB3-32	0.039	0.019	0.95
SJB3-33	0.029	0.069	1.06
SJB3-34	0.055	0.052	1.303
SJB3-35	0.015	0.026	1.256
SJB3-36	0.02	0.038	1.287
SJB3-37	0.141	0.105	1.537
SJB3-38	0.069	0.027	0.919
SJB3-39	0.029	0.03	1.372

ELISA results of antibody recognitions between AK1, AK2 and AK3.

Table 2. Isotypes of established monoclonal antibodies.

Name	Isotype	
	Heavy chain	Light chain
SJB3-31	IgG_1	κ
SJB3-32	IgG_{2b}	κ
SJB3-33	IgG_1	κ
SJB3-34	IgG_{2b}	κ
SJB3-35	IgG_{2b}	κ
SJB3-36	IgG_1	κ
SJB3-37	IgG_1	κ
SJB3-38	IgG_{2b}	κ
SJB3-39	IgG_{2b}	κ

Antibody Subclasses and Affinity

Each of monoclonal antibodies of SJB3-31 through 39 was purified from the mouse ascites and then an experiment was conducted using the mouse typer sub-isotyping kit (Pierce). All nine clones contained κ light chains and IgG_1 or IgG_{2b} heavy chains (Table 2). There are five main antibody classes, designated as IgM, IgD, IgG, IgA and IgE, IgM and IgG antibodies of which are present at the highest percentage in vivo. For the antibody production by antigen immunity, the antibody production pattern varies according to how many times the immunity applies and how long the immunity lasts. When an antigen is first introduced into the body, antigen-specific B clones are selected and then activated to secrete antibodies. IgM antibodies first appear in serum approximately five days after antigen introduction, and IgG antibodies start to appear one or two days later. IgM antibodies last in serum about one week longer than IgM and reach the highest level about three weeks after antigen introduction, and ultimately the in vivo proportion of IgG gets higher. This immunologic principle explains why IgG-type antibodies are mainly generated during the preparation of monoclonal antibodies. IgM antibodies of pentameric structure are sometimes produced and there are difficulties in identifying and applying their characteristics.

Table 3. Dissociation constants (K_d) of monoclonal antibodies.

Name	K_d (M)
SJB3-31	1.96×10^{-10}
SJB3-32	3.12×10^{-10}
SJB3-33	1.37×10^{-10}
SJB3-34	1.0×10^{-10}
SJB3-35	1.6×10^{-10}
SJB3-36	2.8×10^{-10}
SJB3-37	2.0×10^{-10}
SJB3-38	2.0×10^{-10}
SJB3-39	3.5×10^{-10}

Binding affinity of MAbs can be determined, for example, by the Scatchard's method (Table 3).

Identifying the CDRs of Monoclonal Antibodies

Antibodies different in antigen specificity are also different in the amino acid sequences of the variable regions of light and heavy chains. However, if the antibodies are of the same class, the amino acid sequence of the constant region is identical in all the antibodies even if they have different antigen specificity profiles. Some of the variable regions are called hypervariable region (HV region), and the study found that there are three HV regions each in the heavy chain and in the light chain. Since these HV regions make up the antigen-combining site when antigens are combined with antibodies, they are also called complementary determining region (CDR) and the rest consists of framework regions. The CDRs are in extended finger-like loops on the framework regions, and the loop-shaped projected regions are bound like a key-and-lock combination, forming a complementary structure with antigens. The binding sites of antigen and antibody are complementary in three dimensions, but they are chemically specific to each other and involve different types of reactions including hydrogenous binding, ionic binding and hydrophobic binding. For this reason, in order to examine the antigen-binding site of antibody, we identified the amino-acid sequence of the CDR for each antibody using Kabat's numbering (Table 4). Kabat's numbering, an antibody database created by Kabat *et al*. is widely used to study the amino acid sequence of antibodies. The nucleotide and amino acids sequences of variable regions of heavy-chain and light-chain for MAbs, CDRs were determined. The calculated pI values (4.0-4.9) of CDRs which is very acidic were logically in good correspondence to the basic antigen AK3.

Table 4. Physicochemical characters of CDRs for monoclonal antibodies.

SJB3-31	Region	Amino acid sequence	Charge	Hydrophobicity
Heavy chain	CDR1	GYTFTDYAMH	4.55	0.87
	CDR2	IINTYYGNTSYNQKFKGK	4.87	0.83
	CDR3	NYRYDGAMDF	4.65	0.51
	Net		4.07	0.76
Light chain	CDR1	RSSQSIVHSNGNTYLE	4.93	0.78
	CDR2	KVSNRFS	4.85	0.71
	CDR3	FQGSHVPFT	4.89	0.87
	Net		4.00	0.82
SJB3-32	Region	Amino acid sequence	Charge	Hydrophobicity
Heavy chain	CDR1	GYTFSSYWIE	4.61	1.00
	CDR2	EILPGSGSTDYNEKFKGK	5.20	0.70
	CDR3	SHYYGRSHGNSYYFDS	4.41	0.85
	Net		4.34	0.82
Light chain	CDR1	SASQGISNYLN	4.65	0.72
	CDR2	YTSSLHS	4.95	1.17
	CDR3	QQNSKLPFT	4.93	0.93
	Net		4.36	0.89
SJB3-33	Region	Amino acid sequence	Charge	Hydrophobicity
Heavy chain	CDR1	GFTFSGYALS	4.77	0.42
	CDR2	SISRGGNTYYLDSVKGRF	4.68	0.64
	CDR3	EGIYYDYDVGDYHVMDY	4.58	0.54
	Net		4.34	0.71
Light chain	CDR1	SASSSVSYMY	4.61	0.72
	CDR2	RTSNLAS	4.72	1.11
	CDR3	QQYHSYPT	4.90	0.95
	Net		4.34	0.90
SJB3-34	Region	Amino acid sequence	Charge	Hydrophobicity
Heavy chain	CDR1	GYTFTNYYIH	4.50	1.31
	CDR2	WIYPGIVKTKYNEKFKDK	4.95	0.56
	CDR3	GFTTGFAY	4.70	0.36
	Net		4.36	0.77
Light chain	CDR1	RASESVDIFGNSFMH	4.46	0.63
	CDR2	LASNLES	4.78	1.03
	CDR3	QQNNEDPIFT	4.93	1.01
	Net		3.99	0.83
SJB3-35	Region	Amino acid sequence	Charge	Hydrophobicity
Heavy chain	CDR1	GYTFTDFAMH	4.55	0.79
	CDR2	IISTYYGDARYNQKFKGK	4.90	0.65
	CDR3	NYRYDGAMDY	4.61	0.70
	Net		4.06	0.69
Light chain	CDR1	RSSQSIVHSYGNTYLE	4.89	0.75
	CDR2	KVSNRFS	4.85	0.71
	CDR3	FQGSHVPFT	4.92	0.75
	Net		4.21	0.78
SJB3-36	Region	Amino acid sequence	Charge	Hydrophobicity
Heavy chain	CDR1	GYSFTGYTMN	4.52	0.98
	CDR2	LINPYNGAISYSQKFRGK	4.83	0.72
	CDR3	GGFYYGYDWYFDV	4.55	0.44
	Net		4.37	0.76
Light chain	CDR1	KSSQSLLDSDGKTSLS	4.78	0.63
	CDR2	LVSKLDS	4.85	0.53
	CDR3	CHFPQT	4.95	0.86
	Net		4.39	0.71

Table 4. Continued.

SJB3-37	Region	Amino acid sequence	Charge	Hydrophobicity
Heavy chain	CDR1	GYSFTGYTMN	4.52	0.98
	CDR2	LINPYNGAISYSQKFRGK	4.83	0.72
	CDR3	GGFYYGYDWYFDV	4.55	0.44
	Net		4.36	0.77
Light chain	CDR1	KSSQSLLDSDGKTSLS	4.78	0.63
	CDR2	LVSKLDS	4.85	0.53
	CDR3	CHFPQT	4.95	0.86
	Net		4.04	0.71
SJB3-38	Region	Amino acid sequence	Charge	Hydrophobicity
Heavy chain	CDR1	GFTFSSYAMS	4.78	0.61
	CDR2	TISSGGSYTYYPDSVKG	4.72	0.59
	CDR3	TGNYFDY	4.65	0.73
	Net		4.02	0.77
Light chain	CDR1	RSSQSIVHSNGNTYLE	4.93	0.26
	CDR2	KVSNRFS	4.85	0.23
	CDR3	FQGSHVPFT	4.92	0.23
	Net		4.02	0.24
SJB3-39	Region	Amino acid sequence	Charge	Hydrophobicity
Heavy chain	CDR1	GFTFSSYAMS	4.78	0.61
	CDR2	TISSGGSYTYYPDSVKG	4.72	0.59
	CDR3	TGNYFDY	4.65	0.73
	Net		4.02	0.79
Light chain	CDR1	RSSQSIVHSNGNTYLE	4.93	0.26
	CDR2	KVSNRFS	4.85	0.23
	CDR3	FQGSHVPFT	4.92	0.23
	Net		4.02	0.24

Table 5. Summary of the mimotopes for the monoclonal antibodies aganst AK3.

mAb	Epitope peptide
SJB3-31 SJB3-34 SJB3-39	E H Q T R E L P P P N
SJB3-33 SJB3-35 SJB3-36	K S L S R H D H I H H H
SJB3-32 SJB3-37	G H I H S M R H H R P T
SJB3-38	D N A N S S I R S H T Y

Epitope Mapping

Using the random peptide library (Ph.D.-12TM Phage display library kit, NEB) derived from recombinant M13mp19 carrying a minor coat protein pIII gene Modifred to have an additional 12 amino acids in its transcript by genetic engineering, epitope mapping was carried out by selecting pahges having a high affinity to the MAbs to adenylate kinase isozyme, known as biopanning technique. Biopanning was performed according to the following procedure, in which further rounds of biopanning were carried out up to a minimum four times. The selected phages by biopanning using the AK3-specific MAbs were diluted and plated in solid agar plate. Single-stranded (ss) DNA was isolated from the cultures phages by PEG precipitation, and analyzed for DNA sequence. As a result, mimetic peptides as listed Table 5.

Conclusions

Myocardial infarction can lead to death in a very short time once it develops, so the condition requires an earlier diagnosis and procedure. Although there are many clinical diagnostic parameters available, any of them is not ideal to diagnose myocardial infarction by itself and in a timely manner. AK3 is limitedly present in the heart muscle and liver and only discharged into blood by myocardial necrosis. AK3 is detectable earlier, and thus it is likely to become a new biochemical parameter for diagnosis of myocardial infarction.

Today monoclonal antibodies are frequently used for diagnosis and research because of its advantage of specificity, compared to polyclonal antibodies. That's why we undertook the preparation of monoclonal antibodies specific to AK3. Unlike polyclonal antibodies, monoclonal antibodies are homogeneous antibodies that can be semi-permanently produced from a single clone of cells. These antibodies provide an im-

mediate response with high antigen-binding specificity and are relatively low in cross-reactivity.

In this study, we found a high rate of monoclonal antibody production by hybridoma cells, and for the hybridoma cell line for the generation of nine monoclonal antibodies their reactivity to antigens was highly stable at 100 percent. In consequence, the prominent activity and stability of those antibodies suggest their availability as a diagnostic parameter. In the way of collecting ascites via abdominal injection, we purified the antibodies in large quantities to identify their biochemical properties. As a result we successfully established the production of nine AK3-specific monoclonal antibodies whose affinity for rAK3 is very high at K_d=~10^{-10} M. Those antibodies showed extraordinary specificity compared to other existing monoclonal antibodies, when we tested them for cross-recognition. As the respective monoclonal antibodies recognized linear epitopes as well as the structural nature of epitopes, their excellent analytical ability was verified both in ELISA and on immunoblots according to their respective properties. An experiment for sequence analysis of the antibodies was also conducted. In this experiment, the primer used to amplify antibody genes is designed based on Kabat database and still remains secured because it corresponds to the base of recombinant antibody. The sequence analysis of those monoclonal antibodies revealed that their overall net charge was negative. This corresponds with the characteristics of AK3 with a PI of 12. There is a report that the monoclonal antibodies being currently employed for diagnosis generate false signals due to the nonspecific interaction of serum proteins including human serum albumin and anti-animal antibodies. This problem provides a significant obstacle which affects the reliability of the diagnostic kit in direct visual decision making. The use of recombinant antibodies is a way to solve the problem. This can significantly reduce the nonspecific interactions with antibodies or other similar substances in structure by removing the Fc region of each antibody. If the monoclonal antibodies produced for this study are used as direct diagnostics, this recombinant antibody technology will be usefully applicable.

Materials and Methods

Immunization of Mice and Generation of Monoclonal Antibodies (MAbs)

After emulsifying 0.16 mL of recombinant AK3 protein (SJ biomed Inc., Ansan, Korea) solution of 200 μg, with an equal volume of complete Freun's adjuvant, the resulting emulsion was intraperitoneally injected three times into BALB/c mice (6 week old) in intervals of 3 weeks. In the case of second and third injection, incomplete Freund's adjuvant was used. The final boosting was carried out by intravenous injection on the tail of the mice.

After the mice were examined to produced to antibodies specific to the injected antigen AK3 by ELISA test using mouse serum, the spleen was aseptically excised from the immunized mice, and dispersed in DMEM (Dulbecco's Modified Eagle's Medium, Gibco BRL) medium containing fetal bovine serum (Gibco BRL) and 0.5% gentamycin (Gibco BRL), thus giving a splenocyte dispersion solution. The splenocytes were fused with the already prepared SP2/O-Ag14 myeloma cells (American Type Culture Collection, Rockville, MD) from the same mice strain by using PEG, as follows.

The fused cells were harvested in HAT medium for 4 weeks and after this, the cells were harvested in HT medium. Screening for antibody production was performed using an ELISA assay in microtiter wells coated with AK3 (100 ng/well) recombinant protein.

Absorbance was measured at 490 nm. After selecting wells showing of absorbance of over 0.5, the colonies in the ELISA-positive wells were transferred to 0.5 mL-scale culture. Then, cloning of hybridoma cells was carried out by a limiting dilution method.

By ELISA, hybridoma cells secreting anti-AK3 monoclonal antibodies were selected.

The cloning process was carried out three times.

MAbs from the selected clones were purified from culture supernatants of fused cells cultured in FBS containing DMEM medium under 5% CO_2 and ascitic fluids from BALB/C mice injected with pristine and intraperitoneally injected with 1×10^7 hybridoma cells in a good state to induced tumor in their abdominal cavity.

MAbs produced were purified by affinity chromatography using protein A resins (Amersham Biosciences, Uppsala, Sweden).

Assay for Cross-reactivity of MAbs with AK1 and AK2

Same concentration of AK1, AK2 and AK3 was aliquotted to 96-well microtiter plates, followed by incubation at 4°C for 24 hrs to allow coating of the wells with the proteins. In this coating step, 0.05 M bicarbonate buffer of pH 9.6 was used. Then, each well was washed with PBS containing 0.05% Tween 20. After washing, 300 μL of PBS 0.5% casein, 0.02% sodium azide and 0.05% Tween 20 was added to each well, and the plate was incubated at 37°C for 2 hrs. After blocking, each well was washed with PBS containing

0.05% Tween 20.

Same volumn of hybribomas, transferred to 48-well plates and cultured in 1 mL of HAT medium therein for 1 day, added to each well coated with AK1,AK2 or AK3. After incubation at 37°C for 2 hrs, each well washed with PBS containing 0.05% Tween 20.

100 μL of 1 : 1000 dilution of a goat anti-mouse IgG (Fc specific)-HRP-conjugated antibody (Pierce) in PBS was added to each well, followd by incubation at 37°C for 1 hr 30 min. After washing, absorbance at 490 nm was measured using a substrate, OPD.

Ig Subclass Determination

The isotypes of the MAbs were determined by Mouse Typer Sub-Isotyping Kit (Pierce).

Epitope Mapping

Using the random peptide library (Ph.D.-12TM Phage display library kit, NEB) derived from recombinant M13mp19 carrying a minor coat protein pIII gene modified to have an additional 12 amino acids in its transcript by genetic engineering, epitope mapping was carried out by selecting pahges having a high affinity to the MAbs to adenylate kinase isozyme, known as biopanning technique. Biopanning was performed according to the following procedure, in which further rounds of biopanning were carried out up to a minimum four times.

Affinity Determinations

The affinity of the MAbs were determined according to solide-phaseimmunoassay. In order to obtain a degree of dissociation, a saturation concentration and a low range concentrarion of an antigen were determined.

The affinities were calculated using the Scatchard method.

Sequence Analysis of Variable Regions of Antibodies and Determination of CDR

Isolation of total RNA form hybridoma cells. Total RNA was prepared using an RNA Mini Kit (Qiagen). Then synthesys of cDNA was carried out using a cDNA cycle^{TM} kit (Invitrogen). The variable region of antibodies were amplified using DNA Thermal cycler 480 (Perkinelmer). The heavy chain and light chain primers used in the PCR reaction originate from a portion of FR1 and the conserved regions of the currently identified mouse antibodies (mouse ScFv module, Amersharm Pharmacia Biotech).

Obtained PCR products was mixed pCLTA1 DNA and ligation was carried out at 16°C overnight. The ligation mixture was transformed JM 109 competent cells. Plasmid DNA was isolated from the cultured cells. Then, isolated plasid DNA was digested with restriction enzymes. The variable region DNA fragments with correct restriction enzyme mapping results were futher analyzed by DNA sequencing. From the DNA and amino acid sequences of the variable regions, CDRs were determined using Kabat Numbering Scheme.

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