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Declarations under Rule 4.17:

- as to applicant's entitlement to apply for and be granted a
patent (Rule 4.17(ii))

(54) Title: PRODUCTION OF MONOCLONAL ANTIBODY SPECIFIC TO CELL RECEPTOR CD24

(57) Abstract: A monoclonal antibody that binds to the 27th-59th amino acids of CD24 surface receptor protein with high affinity is synthesized by means of the present invention. A therapeutic CD24 monoclonal antibody that can be used as a cancer stem cell marker to reduce the survival of cells of many types of cancer, especially breast cancer, was synthesized. In addition, it is understood that the CD24 12E9 antibody used has no toxic effect on normal blood cells.

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Production Of Monoclonal Antibody Specific To Cell Receptor CD24

5 Technical Field of the Invention

The present invention relates to a novel monoclonal antibody that has therapeutic effects, and that is capable of binding to the CD24 surface marker with high affinity.

State of the Art

CD24 is found in bone marrow, colon, endocrine system, and chondrocyte cells. Moreover,
10 scientific studies have indicated that it is responsible for tumor induction and resistance in many cancer types, particularly in breast cancer as a cancer stem cell marker. Therefore, a therapeutic CD24 monoclonal antibody was synthesized to be used in reducing the survival of cancer cells in the cancer types that belong to the abovementioned systems.

It was determined with the present invention that the 12E9 monoclonal antibody specific to
15 CD24 reduces the viability of glioblastoma cells in vitro, is not toxic in vitro and in vivo. Anti-CD24 12E9 antibody was produced by hybridoma technology.

Technical Problems the Invention Seeks to Solve

It is possible to realize cheap, safe, and easy production of cells that produce monoclonal antibodies by means of the hybridoma technology with the present invention. Division of the
20 cells that produce antibody is provided to continue with the fusion of spleen/lymph cells taken from mice which are immunized and whose antibody response is determined, with the mouse myeloma cells with this technology. The hybrid cells obtained with the invention are stored within liquid nitrogen. When it is needed, frozen cells can be thawed and used for many years by means of culturing the same. There is a potential for producing the same very economically.
25 It can be used in Medical Biology, Molecular Biology, and Genetics, Oncology.

Description of the Invention

It is aimed to produce monoclonal antibody specific to CD24 protein and in this direction, immunization of the mice with CD24 peptide, studies in terms of monoclonal antibody production, purification, and characterization of the same were performed. It was realized to

5 use the same in glioblastoma tumors in mice where death occurs in glioblastoma cells to which produced CD24 antibody was applied in vitro.

Detailed Description of the Invention

12E9 monoclonal antibody specific to CD24 was produced by using the hybridoma technology. The hybridoma technique is a method that is based on fusing the splenic lymphocytes of the
10 animal that produce active antibody for the production of a monoclonal antibody with myeloma (cancer) cells. As a result of this process, immortal hybrid cells producing monoclonal antibody (Ab) that are devoted to a single determinant group (epitope) of an antigen are achieved.

Producing monoclonal antibody specific to CD24 protein is provided with the studies of immunization of the mice with CD24 peptide, producing the monoclonal antibody, purification,
15 and characterization of the same.

The first step for the production of a monoclonal antibody is to achieve an immune response specific to the target molecule. For this reason, a peptide that covers the section between 27th-59th amino acids of CD24 antigen that is produced recombinantly as an immunogen in the production studies of monoclonal antibody against CD24. CHO cells were preferred for the
20 production of the peptide since there were many glycosylation sites in the CD24 antigen. The cloning strategy used to produce antigens is as follows:

In the invention, 4, 8-week-old female BALB/c mice species were used. The mice were immunized with antigens 4 times at 2-week intervals. For the first immunization, Complete Freund's Adjuvant (CFA) equal to an amount of 50µg antigen was mixed and was injected into
25 each mouse intraperitoneally (IP). The following immunizations were made by mixing 50 µg of antigen with an equal amount of Incomplete Freund's Adjuvant (IFA). The measurement of the antibody responses formed in mice as a result of immunizations was provided by indirect ELISA. Determining the antibody level by ELISA method was performed as follows:

- Each well of ELISA plates with 96-well was coated with 250-500ng/well CD24 antigen
30 and kept for 1 night at 4°C with a closed cover.
- The next day, the wells washed with 0.2% Tween-PBS were blocked for 1 hour with 200 µl of 1% milk powder (w/v in PBS).

- 5 - After the wells are washed with 0.2% Tween-PBS again, the diluted serum received from the mice in a ratio of 1/100, 1/1000, and 1/5000 was placed in the wells (100µl /well) and was kept at 37°C for 1 hour.
- After washing the wells with 0.2% Tween-PBS, AP-conjugated secondary antibody developed against mouse antibodies was diluted in a ratio of 1/3000 and was added into
10 the wells (100 µl /well). Plates are kept at 37°C for 1 hour.
- After the wells were washed with 0.2% Tween-PBS 5 times, the substrate (4-nitrophenyl phosphate) was prepared such that it had a concentration of 1mg/ml (substrate buffer was dissolved in 1mM ZnCl₂, 1mM MgCl₂, 0.1M glycine dH₂O and pH was adjusted to 10.4, total volume was 200 ml) and 100 µl was added per each well.
- 15 - After 30-60 minutes the absorbance (OD) was read at 405 nm.

ELISA measurements were made as of the second immunization. The base was coated with 250-500 ng / well CD24 antigen in these studies. Obtained mice serums were tested by diluting the same in a ratio of 1/100, 1/1000.

It was decided to fuse mice with OD⁴⁰⁵ nm value greater than 2, with high and specific antibody
20 response as a result of the ELISA test and for this aim, 4 days before the fusion, a booster immunization was realized with 25 µg antigen without adjuvant from the tail in the same concentration. The stimulation of the cells producing antibodies against the antigen and their increase in numbers are aimed at the booster process.

Fusion and Hybridoma Selection

25 Hybridoma technology was used for monoclonal and in-vitro production of B cells that have anti-CD24 antibody production capacity. Division of the cells that produce antibody is provided to continue with the fusion of spleen/lymph cells taken from mice which are immunized and whose antibody response is determined, with the mouse myeloma cells with this technology.

30 After deciding to perform a fusion, the myeloma cells to be used in the fusion were removed from the liquid nitrogen and placed in the culture and were reproduced to 200-300 million by performing passage every other day, at least 10 days before the planned fusion. Moreover, 1 day before the fusion, macrophages that were not immunized and were taken from the abdominal cavity of another mouse were placed on the plates on which cells would be placed after the fusion, and the medium was enriched for hybridoma culture. After the spleen/lymph

5 cells from the mice with positive immune response were isolated on the day of fusion were washed and mixed with the counted myeloma cells with determined ratios. The fusion of cells was realized by slowly adding PEG 4000 to this mixture. Then respectively;

- Only DMEM (Dulbecco's Modified Eagle Medium),
- DMEM with 20% added serum
- 10 - DMEM with 20% added serum, 0.1% Gentamicin, 2% HAT (hypoxanthine-aminopterin-thymidine) was added.

HAT was used in the culture for the selection of hybridizing cells after fusion. HAT causes the death of myeloma cells with HGPRT mutation used in the fusion that do not fuse with the spleen cells. Spleen B lymphocytes and hybrid cells that can perform pyrimidine and purine synthesis
15 will continue to survive in these selective conditions. However, spleen B lymphocytes that cannot make hybridization with the myeloma cells will die within 4-5 days under normal conditions. Therefore, only myeloma-spleen/lymph B lymphocyte hybrids will survive for a long time.

Screening the formation of hybrid clones on the plates is realized 10 days after the fusion and
20 the plates on which clone formation was being observed were tested with ELISA for the presence of antibody on the plates. HT containing medium was added on the plates instead of the removed supernatant and after 10 days the wells of the clones with antibody response were marked with the ELISA test for the 2nd time.

The fusion study was realized with the mice that were detected to give specific antibody
25 responses. The fusion cells that were fed with a selective medium in the fusion study performed were distributed to the plates on which macrophages were placed previously and after 15 days they were examined under the microscope. As a result of the examinations made, an indirect Elisa test was applied to the clones by marking the ones that reached a determined size (with the supernatant in the well where there were $\approx 1056/156$ clones) and it was observed that there
30 were 8 responses in total against CD24 coated in the Elisa plate well. Clone screening was performed after 3 days and this time clones that were small in the previous screening but reached a certain density in the second screening were also included in the ELISA test. For CD24, which was positive as a result of ELISA, it was determined that it did not resulted in crossing in the test performed in the ELISA well that was not antigen-coated (only blocked with
35 milk powder) for the 12e9 clone. As a result of this test, it was determined that the hybrid clone

5 in the 12E9 well was CD24 specific, and following the increase in the number of cells, spares were prepared to the adjacent plate wells that are known to be empty. After fusion, dilution was conducted in 12E9 hybrid cells which were a clone giving response to CD24 so as to form a strain consisting of a single clone. On the 19th day of the fusion, macrophages that were not immunized and were taken from the abdominal cavity of another mouse were placed on the
10 plates on which subsequently cells of 12E9 clones would be placed after the fusion and the medium was enriched for hybridoma culture. On the 20th day of the fusion, 12E9 hybrid cells were taken into tubes with the help of a pipette and cell count was carried out and distributed as a single cell into 4 pieces of 96-well culture plates. As a result of 1-week follow-up, ELISA studies were realized with the clone consisting of a single cell and it was seen that the clone
15 gave a strong response and did not respond when ELISA was carried out in only the presence of PBS without antigen, and the studies of cell culture and accumulation of supernatant were initiated. In addition to these studies, some of the cells of the 12E9 clone that were dropped individually were frozen and backed up in liquid nitrogen.

Characterization of Anti-CD24 Antibody

20 It was characterized by using isotyping strips and ELISA so as to determine the type of antibody Immunoglobulin (Ig) produced by the clone named 12E9 determined to produce a reactive antibody with CD24. As a result of the characterization, the isotype of the antibody named 12E9 was determined as IgG1.

Purification of Antibody

25 Protein A was purified by an affinity chromatography method because the obtained antibody is IgG isotype. Immunoaffinity chromatography is one of the most commonly used methods on Protein A or Protein B bound solid phase so as to separate mAb's with IgG isotype from serum proteins and IgGs. Protein A and Protein G are the bacterial proteins that can specifically bind to the Fc section of IgG class mouse immunoglobulins that do not bind to an antigen. While
30 purification was made with Protein A; antibodies in the mixture were bind to the column and the other proteins were washed and removed. Then, bound antibodies are collected with the change of pH of the medium. The process of purification of antibodies was performed in two steps. The antibodies that are concentrated by being precipitated with AS in the first step were purified with Protein A affinity chromatography. Antibodies were loaded onto Protein A
35 immobilized column within the binding buffer. After the loading process, the column was

- 5 washed with binding buffer and unbound foreign molecules were removed, antibodies bound to the column were removed with the elution buffer so as to obtain pure antibody.

Table 1. Purification Scheme of Monoclonal antibody (12E9) specific to CD24.

	12E9 Antibody developed against CD24 Protein
Antibody Isotype	IgG1
Column Type	Protein A
Binding Buffer	1.5 M Glycine/NaOH buffer, 3 M NaCl, pH 8.0
Elution Buffer	0.1 M Glycine pH 2.7
Balance Buffer 75ul was added on 1ml elute	1M Tris pH 9
Dialysis	Dialyzed against 1XPBS. Buffers were changed every 2 hours, one during the night
Flow speed (purification)	1ml/min
The amount loaded onto the colon	3 ml sample + 3 ml binding buffer

- 50 µl Tris, pH 9 was added to each of the tubes in which elution fractions were collected so as to prevent activity loss of the eluted antibodies at low pH. The amount of protein taken from the column was evaluated by absorbance reading at 280 nm, the activity at the fractions was evaluated by indirect ELISA. Since purified antibodies subject to aggregation in water, antibodies were dialyzed against PBS and were frozen at -80 °C and were lyophilized until they are completely dried. Lyophilized antibodies were stored at +4°C.
- 10

CLAIMS

5 **1.** A synthesis method of a monoclonal antibody that binds to the 27th-59th amino acids of CD24 surface receptor protein with high affinity, characterized in that; it comprises the following production steps:

- Immunizing 4, 8-week-old female BALB/c mice species,
- Performing fusion and Hybridoma Selection,
- 10 - Performing characterization of Anti-CD24 Antibody,
- Purifying antibodies,
- Freezing purified antibodies at -80°C and lyophilizing the same until they are completely dried,
- Keeping Lyophilized antibodies at +4C.

15

2. A peptide used for CD24 according to claim 1, characterized in that; it is EcoRI-Kozak Sequence-Artificial signal peptide-hIG1Fc-Linker1-FLAG / EK-CD24 (27-59) -Stop codon-HindIII peptide

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25

INTERNATIONAL SEARCH REPORT

International application No.

PCT/TR2020/050972

A. CLASSIFICATION OF SUBJECT MATTER

A61K 39/395 (2006.01)i; C07K 16/28 (2006.01)i; C07K 16/30 (2006.01)i; G01N 33/53 (2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K; C07K; G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CN 103819561 A (UNIV CHINA PHARMA) 28 May 2014 (2014-05-28) Embodiment 1	1-2
X	CN 108373504 A (GRACELL BIOTECHNOLOGY SHANGHAI CO LTD) 07 August 2018 (2018-08-07) The Whole Document.	1-2
A	HOLZLOHNER, P. & HANACK, K.: "Generation of Murine Monoclonal Antibodies by Hybridoma Technology", J. Vis. Exp. (119), e54832, DOI:10.3791/54832, 1 February 2017 (2017-02-01), The Whole Document.	1-2

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

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Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

a. ☒ forming part of the international application as filed:

☐ in the form of an Annex C/ST.25 text file.
☒ on paper or in the form of an image file.

b. ☐ furnished together with the international application under PCT Rule 13^{ter}.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

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☐ on paper or in the form of an image file (Rule 13^{ter}.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.3. Additional comments:

Contrary to the requirements of Rule 13 ter. 1, the amino acid sequence was disclosed only in the written format as "Sequence-Artificial signal peptide-hlG1Fc-Linker1-FLAG / EK-CD24 (27-59)–Stop codon-HindIII peptide".

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/TR2020/050972

Patent document cited in search report			Publication date (day/month/year)	Patent family member(s)			Publication date (day/month/year)
CN	103819561	A	28 May 2014	CN	105646712	A	08 June 2016
				CN	105646712	B	12 July 2017
				CN	105646713	A	08 June 2016
				CN	105646713	B	12 July 2019
CN	108373504	A	07 August 2018	NONE			