



(12) DEMANDE DE BREVET CANADIEN
CANADIAN PATENT APPLICATION

(13) A1

(86) Date de dépôt PCT/PCT Filing Date: 2019/02/05
(87) Date publication PCT/PCT Publication Date: 2019/08/08
(85) Entrée phase nationale/National Entry: 2020/08/04
(86) N° demande PCT/PCT Application No.: JP 2019/004123
(87) N° publication PCT/PCT Publication No.: 2019/151539
(30) Priorité/Priority: 2018/02/05 (JP2018-018652)

(51) CI.Int./Int.Cl. C07K 19/00 (2006.01),
A61K 38/47 (2006.01), A61K 47/68 (2017.01),
A61P 21/00 (2006.01), C07K 16/28 (2006.01),
C07K 16/46 (2006.01), C12N 9/26 (2006.01),
C12N 9/40 (2006.01), C12N 15/13 (2006.01),
C12N 15/56 (2006.01), C12N 15/62 (2006.01)

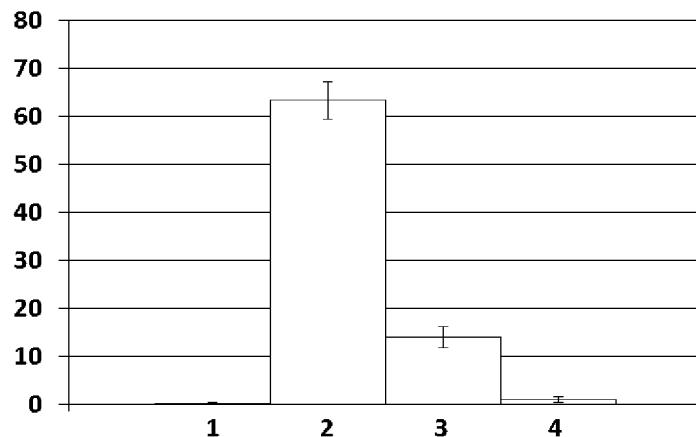
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(54) Titre : PROCEDE D'ADMINISTRATION D'UN MEDICAMENT A UN MUSCLE

(54) Title: METHOD FOR DELIVERING DRUG TO MUSCLE



(57) Abrégé/Abstract:

[Problem] To provide a method for efficiently incorporating a drug that is to function in muscle tissue, but when administered to an organism is itself not adequately incorporated into muscle tissue, into muscle tissue, particularly into muscle tissue constituted of skeletal muscle or heart muscle. [Solution] A complex of a drug and an anti-human transferrin receptor antibody, wherein the drug is a drug having a physiological activity that is to exhibit functionality in muscle tissue, for example, a lysosomal enzyme such as acidic α -glucosidase or α -galactosidase A.

ABSTRACT

[Problems] To provide a technique for efficiently incorporating an agent having to function in muscle tissue, which is not sufficiently incorporated into muscle tissue when administered in body, into muscle tissue, particularly muscle tissue composed of skeletal muscle or cardiac muscle. [Solution] A conjugate of an anti-human transferrin receptor antibody and an agent, wherein the agent is a biologically active agent that should function in muscle tissue, e.g., a lysosomal enzyme such as acid α -glucosidase, α -galactosidase A.

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DESCRIPTION

Title of Invention

METHOD FOR DELIVERING DRUG TO MUSCLE

Technical Field

5 [0001] The present invention relates to the delivery of a pharmaceutical agent to muscle, that agent shall exert its physiological activity in muscle, and more particularly to a conjugate comprising a desired pharmaceutical agent coupled to an anti-transferrin antibody, and a method of administering the conjugate to cause the desired agent to exert its bioactivity in muscle.

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Background Art

15 [0002] Recently, enzyme replacement therapy for lysosomal disease caused by functional deficiency of lysosomal enzymes has been established, and the patient's vital prognosis and quality of life (QOL) have improved. Drugs containing lysosomal enzymes as active ingredients used in enzyme replacement therapy include a drug for glycogen storage disease type 2 (Pompe disease) containing human acid α -glucosidase (hGAA) as the main agent, a drug for mucopolysaccharidosis type 2 (Hunter disease) containing human iduronate-2-sulfatase (hI2S) as the main agent, a drug for Fabry disease containing human α -galactosidase A (h α -GalA) as the main agent, and a drug for Gaucher disease containing human glucocerebrosidase as the main agent.

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25 [0003] Lysosomal enzymes administered to patients in enzyme replacement therapy are taken up into cells and exert their functions. For example, human acid α -glucosidase (hGAA), human

iduronate-2-sulfatase (hI2S), and human α -galactosidase A (h α -GalA), modified by an N-type sugar chain containing mannose-6-phosphate (M6P), are taken up into cells via binding of M6P to M6P receptor. Human glucocerebrosidase, a drug for Gaucher disease, is also modified by N-type sugar chain, whose reducing end is mannose, and is taken up into cells of the reticuloendothelial system, such as macrophages via binding of mannose to mannose receptors. Regarding the uptake into cells via binding of M6P and M6P receptors, a bis-phosphate type sugar chain containing two M6Ps have 100 times higher affinity for M6P receptor than a mono-phosphate type sugar chain containing one M6P. M6P contained in sugar chain greatly affects the efficiency of the cellular uptake of lysosomal enzymes.

[0004] The human lysosomal enzyme used in enzyme replacement therapy is a recombinant protein produced by expressing a gene encoding a human lysosomal enzyme, the gene introduced into a host cell such as a CHO cell using a gene recombination technology. Recombinant human lysosomal enzymes produced as recombinant proteins need to be appropriately modified by sugar-chain in order to exert their pharmaceutical effect. In other words, it is essential for hGAA, h α -GalA, and hI2S to be modified with the sugar chain containing mannose-6-phosphate, and for human glucocerebrosidase to be appropriately modified with the sugar chain whose reducing end is mannose, to exert their pharmaceutical effect.

[0005] In the case of recombinant human acid α -glucosidase (rhGAA), the main target organ where its pharmaceutical effects shall be exerted is muscle, particularly skeletal muscle. However, the number of M6P

5 contained in sugar chains is 1 or less on average per molecule in rhGAA used for the enzyme replacement therapy, so that a large part of the sugar chains is mono-phosphate type, and the sugar-chain modification by M6P is not satisfactory (Non-Patent Document 1). Therefore, as rhGAA is not incorporated in the target organ inefficiently, the dose per administration is extremely high, i.e. 20mg/kg. Depending on the patient's symptoms, the dose may be increased to 40mg/kg, but still the enzyme administered is not considered to be fully functional.

10 [0006] A method using a ligand-receptor system other than M6P-M6P receptor has been developed as a method for efficient uptake of rhGAA into a target organ. For example, it is known that a fusion protein obtained by fusing RAP (receptor-associated protein), which is a ligand of a low-density lipoprotein receptor family (LDL receptor), and hGAA is taken up into cells in an M6P-independent manner in experiments 15 using cultured cells (Non-Patent Document 2). According to this method, even rhGAA insufficiently modified by the sugar chain containing M6P can be taken up into cells via the binding of RAP and its receptor.

Citation List

20 **Non Patent Literature**

[0007] [Non-Patent Document 1] Zhu Y. et al., Biochem. J. 389, 619-28 (2005)

[Non-Patent Document 2] Prince WS. et al., J Biol. Chem. 279, 35037-46 (2004)

25 **Summary of Invention**

Problems to be Solved by the Invention

[0008] It is an objective of the present invention to provide a method for efficiently incorporating a pharmaceutical agent having to function in a muscle into a muscle when the pharmaceutical agent is administered in vivo, and its related art.

5 **Means for Solving the Problems**

[0009] In the study directed to the above-mentioned purpose, as a result of intensive investigation, the present inventors have found that recombinant human acid α -glucosidase, which should exert its pharmaceutical effect in the muscle but is not sufficiently transported to 10 the muscle as it is, is efficiently taken up into the muscle by conjugation with the anti-human transferrin receptor, and completed the present invention. Thus the present invention includes the following.

- 15 1. A conjugate of an anti-human transferrin receptor antibody and a pharmaceutical agent, wherein the agent has a physiological activity to be exerted in muscle.
2. The conjugate according to 1 above, wherein the anti-human transferrin receptor antibody is a Fab antibody, a $F(ab')_2$ antibody, or a $F(ab')$ antibody.
- 20 3. The conjugate according to 1 above, wherein the anti-human transferrin receptor antibody is a single-chain antibody selected from the group consisting of scFab, sc $F(ab')$, sc $F(ab')_2$, and scFv.
4. The conjugate according to 3 above, wherein in the single-chain antibody a light chain and a heavy chain of the anti-human transferrin receptor antibody are linked via a linker sequence.
- 25 5. The conjugate according to 4 above, wherein the light chain and the heavy chain of the anti-human transferrin receptor antibody are

linked on the C-terminal side of the light chain via the linker sequence.

6. The conjugate according to 4 above, wherein the light chain and the heavy chain of the anti-human transferrin receptor antibody are linked on the C-terminal side of the heavy chain via the linker sequence.

5 7. The conjugate according to any one of 4 to 6 above, wherein the linker sequence comprises 8 to 50 amino acid residues.

8. The conjugate according to 7 above, wherein the linker sequence comprises an amino acid sequence selected from the group consisting of the amino acid sequence (Gly Ser), the amino acid sequence (Gly Gly Ser), the amino acid sequence (Gly Gly Gly), the amino acid sequence set forth as SEQ ID NO: 3, and the amino acid sequence set forth as SEQ ID NO: 4.

9. The conjugate according to 8 above, wherein the linker sequence comprises 15 amino acid residues in which the amino acid sequence set forth as SEQ ID NO: 4 is consecutively repeated three times.

10. The conjugate according to any one of 1 to 9 above, wherein the anti-human transferrin receptor antibody comprises the amino acid sequence set forth as SEQ ID NO: 22 in the variable region of the light chain and the amino acid sequence set forth as SEQ ID NO: 23 in the variable region of the heavy chain.

25 11. The conjugate of 10 above, wherein the amino acid sequence of the variable region of the light chain has an identity not lower than 80% to the amino acid sequence set forth as SEQ ID NO: 22, and the amino acid sequence of the variable region of the heavy chain has an identity not lower than 80% to the amino acid sequence set forth as SEQ

ID NO: 23.

12. The conjugate of 10 above, wherein the amino acid sequence of the variable region of the light chain has an identity not lower than 90% to the amino acid sequence set forth as SEQ ID NO: 22, and the amino acid sequence of the variable region of the heavy chain has an identity not lower than 90% to the amino acid sequence set forth as SEQ ID NO: 23.

13. The conjugate according to 10 above, wherein 1 to 10 amino acids constituting the variable region of the light chain are substituted with other amino acids relative to the amino acid sequence set forth as SEQ ID NO: 22.

14. The conjugate according to 10 above, wherein 1 to 3 amino acids constituting the variable region of the light chain are substituted with other amino acids relative to the amino acid sequence set forth as SEQ ID NO: 22.

15. The conjugate according to 10 above, wherein 1 to 10 amino acids constituting the variable region of the heavy chain are substituted with other amino acids relative to the amino acid sequence set forth as SEQ ID NO: 23.

16. The conjugate according to 10 above, wherein 1 to 3 amino acids constituting the variable region of the heavy chain are substituted with other amino acids relative to the amino acid sequence set forth as SEQ ID NO: 23.

17. The conjugate according to 10 above, wherein 1 to 10 amino acids constituting the variable region of the light chain are substituted with other amino acids relative to the amino acid sequence set forth as

SEQ ID NO: 22, and 1 to 10 amino acids constituting the variable region of the heavy chain are substituted with other amino acids relative to the amino acid sequence set forth as SEQ ID NO: 23.

5 18. The conjugate according to 10 above, wherein 1 to 3 amino acids constituting the variable region of the light chain are substituted with other amino acids relative to the amino acid sequence set forth as SEQ ID NO: 22, and 1 to 3 amino acids constituting the variable region of the heavy chain are substituted with other amino acids relative to the amino acid sequence set forth as SEQ ID NO: 23.

10 19. The conjugate according to 10 above, wherein the anti-human transferrin receptor antibody comprises the light chain comprising an amino acid sequence set forth as SEQ ID NO: 24 and the heavy chain comprising an amino acid sequence set forth as SEQ ID NO: 25.

15 20. The conjugate according to 19 above, wherein the amino acid sequence of the light chain has an identity not lower than 80% to the amino acid sequence set forth as SEQ ID NO: 24, and the amino acid sequence of the heavy chain has an identity not lower than 80% to the amino acid sequence set forth as SEQ ID NO: 25.

20 21. The conjugate according to 19 above, wherein the amino acid sequence of the light chain has an identity not lower than 90% to the amino acid sequence set forth as SEQ ID NO: 24, and the amino acid sequence of the heavy chain has an identity not lower than 90% to the amino acid sequence set forth as SEQ ID NO: 25.

25 22. The conjugate of any of 1 to 21 above, wherein the agent is a peptide or a protein.

23. The conjugate according to 22 above selected from the group consisting of (1) to (4) below:

5 (1) a conjugate in which the protein is linked to the C-terminal side of the light chain of the anti-human transferrin receptor antibody by a peptide bond,

(2) a conjugate in which the protein is linked to the N-terminal side of the light chain of the anti-human transferrin receptor antibody by a peptide bond,

10 (3) a conjugate in which the protein is linked to the C-terminal side of the heavy chain of the anti-human transferrin receptor antibody by a peptide bond, and

(4) a conjugate in which the protein is linked to the N-terminal side of the heavy chain of the anti-human transferrin receptor antibody by a peptide bond.

15 24. The conjugate according to 23 above, wherein the protein is linked to the anti-human transferrin receptor antibody via a linker sequence.

25. The conjugate according to 24 above, wherein the linker sequence consists of 1 to 50 amino acid residues.

20 26. The conjugate according to 25 above, wherein the linker sequence comprises an amino acid sequence selected from the group consisting of a single glycine, a single serine, the amino acid sequence (Gly Ser), the amino acid sequence (Gly Gly Ser), the amino acid sequence set forth as SEQ ID NO:3, the amino acid sequence set forth as SEQ ID NO:4, and the amino acid sequence consisting of 1 to 10 amino acid sequences thereof that are linked consecutively.

27. The conjugate according to any one of 1 to 28 above, wherein the anti-human transferrin receptor antibody is a humanized anti-human transferrin receptor antibody.

5 28. The conjugate according to any one of 22 to 27 above, wherein the protein is a lysosomal enzyme.

29. The conjugate according to 28 above, wherein the lysosomal enzyme is human α -galactosidase A.

10 30. The conjugate according to 29 above, wherein the light chain of the humanized anti-hTfR antibody comprises the amino acid sequence set forth as SEQ ID NO: 24, and wherein the heavy chain of the humanized anti-hTfR antibody is linked on its C-terminal side to the human α -galactosidase A via a linker sequence (Gly Ser), and the whole linked heavy chain has the amino acid sequence set forth as SEQ ID NO: 29.

15 31. The conjugate according to 28 above, wherein the lysosomal enzyme is human acid α -glucosidase.

20 32. The conjugate according to 31 above, wherein the light chain of the humanized anti-hTfR antibody comprises the amino acid sequence set forth as SEQ ID NO: 24, and wherein the heavy chain of the humanized anti-hTfR antibody is linked on its C-terminal side to the human acid α -glucosidase via a linker sequence (Gly Ser), and the whole linked heavy chain has the amino acid sequence set forth as SEQ ID NO: 27.

25 33. A pharmaceutical composition for improving a muscle function comprising the conjugate according to any one of 1 to 32 above.

34. A pharmaceutical composition for ameliorating muscle dysfunction associated with lysosomal disease, comprising the conjugate according to 28 above.

5 35. A pharmaceutical composition for ameliorating muscle dysfunction associated with Fabry disease, comprising the conjugate according to 29 or 30 above.

36. A pharmaceutical composition for ameliorating muscle dysfunction associated with Pompe disease, comprising the conjugate according to 31 or 32 above.

10 37. The pharmaceutical composition according to any one of 33 to 36 above, wherein the muscle is skeletal muscle, cardiac muscle, or smooth muscle.

15 38. Use of the conjugate according to any one of 28 to 32 above for the manufacture of a pharmaceutical composition for ameliorating muscle dysfunction associated with lysosomal disease.

39. Use of the conjugate according to 29 or 30 above for the manufacture of a pharmaceutical composition for ameliorating muscle dysfunction associated with Fabry disease.

20 40. Use of the conjugate according to 31 or 32 above for the manufacture of a pharmaceutical composition for ameliorating muscle dysfunction associated with Pompe disease.

41. Use of the conjugate according to any one of 1 to 32 above as a pharmaceutical composition for improving muscle function.

25 42. A method for delivering a pharmaceutical agent to muscle, comprising a step of producing the agent as a conjugate in which the agent is linked to an anti-human transferrin receptor antibody and a step

of administering the conjugate to an individual.

43. The method according to 42 above, wherein the conjugate is the conjugate according to any one of 1 to 32 above.

5 44. The method according to 42 or 43 above, further comprising a step of improving muscle function of the individual by administration of the conjugate.

45. The method according to any one of 42 to 44 above, wherein the individual has a muscle dysfunction.

10 46. The method according to any one of 42 to 44 above, wherein the individual has a muscle dysfunction associated with lysosomal disease.

47. The method of 46 above, wherein the lysosomal disease is Fabry disease or Pompe disease.

Effects of the Invention

15 [0010] According to the present invention, a pharmaceutical agent exerting its function in a muscle, in particular, a peptide or a protein having a physiological activity can be efficiently delivered to a muscle when the agent is administered in a body by letting the agent to be conjugated with an anti-human transferrin receptor. In other words, by 20 conjugating the agent with the human transferrin receptor, it is possible to provide a more effective therapeutic agent for diseases associated with muscle dysfunction.

Brief Description of Drawings

25 [0011] [Figure 1] A figure showing the quantitative results of glycogen contained in the heart. The quantitative results of the wild-type control group, (1) shows the quantitative results of wild-type control group,

KO-control group, hGAA administration group, and hGAA-anti-hTfR antibody administration group are shown in (1), (2), (3), and (4), respectively. Vertical bars indicate SD values. The vertical axis represents glycogen concentration (mg/g wet tissue weight).

5 [Figure 2] A figure showing the quantitative results of glycogen contained in the diaphragm. The same are represented in (1)~(4), vertical bars, and the vertical axis as in Fig. 1.

10 [Figure 3] A figure showing the quantitative results of glycogen contained in the soleus muscle. The same are represented in (1)~(4), vertical bars, and the vertical axis as in Fig. 1.

[Figure 4] A figure showing the quantitative results of glycogen contained in the tibialis anterior muscle. The same are represented in (1)~(4), vertical bars, and the vertical axis as in Fig. 1.

15 [Figure 5] A figure showing the quantitative results of glycogen contained in the quadriceps femoris muscle. The same are represented in (1)~(4), vertical bars, and the vertical axis as in Fig. 1.

Embodiments for Carrying Out the Invention

20 [0012] In the present invention, the term "muscle" means a tissue mainly composed of skeletal muscle, cardiac muscle, or smooth muscle, and in particular, a tissue mainly composed of skeletal muscle or a tissue mainly composed of cardiac muscle. And the term "muscle tissue" is understood to be synonymous with muscle.

25 [0013] In the present invention, a pharmaceutical agent that should exert physiological activity in a muscle is the agent that cannot exert its physiological activity sufficiently in a muscle for the inefficient transport to the muscle when the agent is administered alone into the

blood. Such agents may be small molecule compounds, peptides, or proteins. Here, the peptide is that to be potentially capable of exerting physiological activity in muscle (bioactive peptide), but alone not to be efficiently transported to muscle when administered in a body. Such a peptide preferably consists of 2 to 100 amino acid residues, e.g., 2 to 50, 5 5 to 100 amino acid residues. Further, such a protein is potentially capable of exerting physiological activity in muscle (bioactive protein), but it alone is not efficiently transported to muscle when administered in a body, and includes lysosomal enzymes such as acid α -glucosidase 10 (GAA) and α -galactosidase A (α -GalA), and so on. Such peptides and proteins may have artificial amino acid sequences or may have amino acid sequences encoded by animal genes. Here, the animal species of the gene encoding the peptide or protein is not particularly limited, but is preferably a mammal, more preferably human, monkey, mouse, 15 domestic animal (horse, pig, sheep, etc.), or pet animal (cat, dog, etc.), and more preferably a human.

[0014] In addition to GAA and α GalA, lysosomal enzymes that need to exhibit functions as medicaments for improving the malfunction of muscle associated with lysosomal diseases include 20 include α -L-iduronidase (IDUA), iduronate-2-sulfatase (IDS), glucocerebrosidase (GBA), β -galactosidase, GM2 activator protein, β -hexosaminidase A, β -hexosaminidase B, N-acetylglucosamine-1-phosphotransferase, α -mannosidase (LAMAN), β -mannosidase, galactosylceramidase (GALC), Saposin C, arylsulfatase 25 A (ARSA), α -L-fucosidase (FUCA1), aspartylglucosaminidase, α -N-acetylgalactosaminidase, acid sphingomyelinase (ASM),

β-glucuronidase (GUSB), heparan N-sulfatase (SGSH), α-N-acetylglucosaminidase (NAGLU), acetyl-CoA: α-glucosaminide N-acetyltransferase, N-acetylglucosamine-6-sulfate sulfatase, acid ceramidase (AC), amylo-1, 6-glucosidase, sialidase, 5 aspartylglucosaminidase, palmitoyl protein thioesterase-1 (PPT-1), tripeptidyl peptidase-1 (TPP-1), hyaluronidase-1, CLN1, CLN2, and a like. These enzymes can be more efficiently delivered to muscle when administered in a body as a form of a conjugate with an anti-human 10 transferrin receptor antibody. Thus, these conjugates can be used as a medicament to ameliorate muscle dysfunction associated with lysosomal disease.

[0015] Iduronate-2-sulfatase (IDS) fused with anti-hTfR antibody can be used as a therapeutic agent for muscle dysfunction in Hunter syndrome, α-galactosidase A fused with anti-hTfR antibody can be used 15 as a therapeutic agent for muscle dysfunction in Fabry disease, α-L-iduronidase (IDUA) fused with anti-hTfR antibody can be used as a therapeutic agent for muscle dysfunction in Hurler syndrome or Hurler-Scheie syndrome, glucocerebrosidase (GBA) fused with anti-hTfR antibody can be used as a therapeutic agent for muscle dysfunction in Gaucher disease, β-galactosidase fused with anti-hTfR 20 antibody can be used as a therapeutic agent for muscle dysfunction in GM1-gangliosidosis types 1-3, GM2 activator protein fused with anti-hTfR antibody can be used as a therapeutic agent for muscle dysfunction in GM2-gangliosidosis AB variant, β-hexosaminidase A fused with anti-hTfR antibody can be used as a therapeutic agent for muscle dysfunction in Sandhoff disease and Tay-Sachs disease,

β-hexosaminidase B fused with anti-hTfR antibody can be used as a therapeutic agent for muscle dysfunction in Sandhoff disease, N-acetylglucosamine-1-phosphotransferase fused with anti-hTfR antibody can be used as a therapeutic agent for muscle dysfunction in I-cell disease, α-mannosidase (LAMAN) fused with anti-hTfR antibody can be used as a therapeutic agent for muscle dysfunction in α-mannosidosis, galactosylceramidase (GALC) fused with anti-hTfR antibody can be used as a therapeutic agent for muscle dysfunction in Krabbe disease, saposin C fused with anti-hTfR antibody can be used as a therapeutic agent for muscle dysfunction in Gaucher-like disease, and arylsulfatase A (ARSA) fused with anti-hTfR antibody can be used as a therapeutic agent for muscle dysfunction in metachromatic leukodystrophy, α-L-fucosidase (FUCA1) fused with anti-hTfR antibody can be used as a therapeutic agent for muscle dysfunction in fucosidosis, aspartylglucosaminidase fused with anti-hTfR antibody can be used as a therapeutic agent for muscle dysfunction in aspartylglucosamiuria, α-N-acetylgalactosaminidase fused with anti-hTfR antibody can be used as a therapeutic agent for muscle dysfunction in Sindler disease and Kanzaki disease, acid sphingomyelinase (ASM) fused with anti-hTfR antibody can be used as a therapeutic agent for muscle dysfunction in Niemann-Pick disease, β-glucuronidase (GUSB) fused with anti-hTfR antibody can be used as a therapeutic agent for muscle dysfunction in Sly syndrome, heparan N-sulfatase (SGSH), α-N-acetylglucosaminidase (NAGLU), acetyl-CoA: α-glucosaminide N-acetyltransferase, and N-acetylglucosamine-6-sulfate sulfatase fused with anti-hTfR antibody

can be used as therapeutic agents for muscle dysfunction in Sanfilippo syndrome, acid ceramidase (AC) fused with anti-hTfR antibody can be used as a therapeutic agent for muscle dysfunction in Farber disease, amylo-1,6-glucosidase fused with anti-hTfR antibody can be used as a therapeutic agent for muscle dysfunction in Cori disease (Forbes/Cori disease), sialidase fused with anti-hTfR antibody can be used as a therapeutic agent for muscle dysfunction in sialidase deficiency, palmitoyl protein thioesterase-1 (PPT-1) fused with anti-hTfR antibody can be used as a therapeutic agent for muscle dysfunction in neuroceroid lipofuscinosis or Santavuori-Haltia disease, tripeptidyl peptidase-1 (TPP-1) fused with anti-hTfR antibody can be used as a therapeutic agent for muscle dysfunction in neuroceroid lipofuscinosis or Jansky-Bielschowsky disease, hyaluronidase-1 fused with anti-hTfR antibody can be used as a therapeutic agent for muscle dysfunction in 10 hyaluronidase deficiency, acid α -glucosidase (GAA) fused with anti-hTfR antibody can be used as a therapeutic agent for muscle dysfunction in Pompe disease, and CLN1 and CLN2 fused with anti-hTfR antibody can be used as therapeutic agents for muscle dysfunction in Batten disease. In particular, the anti-hTfR antibody of 15 the present invention is expected to reach the cerebral parenchyma, hippocampal nerve-like cells, cerebellar Purkinje cells, etc. after crossing the blood-brain barrier, and also to reach nerve-like cells of the cerebral striatum and nigra of the midbrain, so that the pharmaceutical efficacy of the protein can be enhanced by fusing it with a protein to exert its function in these tissues or cells. However, the pharmaceutical 20 use is not limited to use for these diseases.

[0016] In the present invention, the term "lysosomal enzyme" or the term indicating an individual lysosomal enzyme means, in particular, that having a natural amino acid sequence, but is not limited thereto, and as long as it has a lysosomal enzyme, lysosomal enzymes in which 5 mutations such as substitutions, deletions, additions, and a like are introduced to the amino acid sequence of the natural lysosomal enzyme are also included in the lysosomal enzyme. When the amino acids of the amino acid sequence of the lysosomal enzyme are substituted with other amino acids, the number of amino acids to be substituted is preferably 10 from 1 to 10, more preferably from 1 to 5, even more preferably from 1 to 3, and still more preferably from 1 to 2. When the amino acids of the amino acid sequence of the lysosomal enzyme are deleted, the number 15 of amino acids to be deleted is preferably from 1 to 10, more preferably from 1 to 5, even more preferably from 1 to 3, and still more preferably from 1 to 2. A mutation combining such substitution and deletion of amino acids can also be introduced to the lysosomal enzyme. When the amino acids are added to the amino acid, preferably 1 to 10, more 20 preferably 1 to 5, even more preferably 1 to 3, and still more preferably 1 to 2 amino acids are added to the amino acid sequence of the lysosomal enzyme or to N-terminal or C-terminal side of the lysosomal enzyme. A mutation combining such addition, substitution and deletion 25 of amino acids can also be introduced to the lysosomal enzyme. The amino acid sequence of the mutated lysosomal enzyme preferably has an identity not lower than 80%, more preferably 90%, and even more preferably 95% to the amino acid sequence of the original lysosomal enzyme. The same applies to proteins and peptides other than lysosomal

enzymes.

[0017] Here, when it is referred to as “lysosomal enzyme has lysosomal enzyme activity”, it means that the lysosomal enzyme has an activity not less than 3% relative to the activity inherent in the wild type lysosomal enzyme when conjugated with anti-hTfR antibody. However, the activity is preferably not less than 10%, more preferably not less than 20%, even more preferably not less than 50%, and still more preferably not less than 80% relative to the activity inherent in the wild type lysosomal enzyme. The same is applied when lysosomal enzymes conjugated to anti-hTfR antibody are mutated. The same applies to proteins and peptides other than lysosomal enzymes.

[0018] Substitutions of amino acids in the amino acid sequence of peptides or proteins (including lysosomal enzymes and antibodies) with other amino acids include, for example, substitutions between amino acids in the same group, such as aromatic amino acids (Phe, Trp, Tyr), aliphatic amino acids (Gly, Ala, Leu, Ile, Val), amide-type amino acids (Gln, Asn), basic amino acids (Lys, Arg, His), acidic amino acids (Glu, Asp), amino acids with hydroxyl groups (Ser, Thr), branched amino acids (Val, Leu, Ile), amino acids with small side chains (Gly, Ala, Ser, Thr, Met), and amino acids with nonpolar side chains (Ala, Val, Leu, Ile, Pro, Phe, Met). Substitutions with such similar amino acids are predicted not to result in a change in the phenotype of the protein (i.e., conservative amino acid substitutions). Examples of conservative amino acid substitutions are well known in the art and described in various references (see, e.g., Bowie et al., *Science*, 247:1306-1310 (1990)).

[0019] In the present invention, "homology" or "identity" means the

ratio (%) of homologous amino acid residues to total amino acid residues in the optimal alignment when two amino acid sequences are compared using a homology calculation algorithm. It has been well known in the art of the invention to compare the two amino acid sequences by a homology (identity) shown by such a ratio and may be readily understood by those skilled in the art. In the present invention, as a homology calculation algorithm for calculating homology between the amino acid sequence of the original protein (including an antibody) and the amino acid sequence of the mutated protein, BLAST (Altschul 5 SF. J Mol. Biol. 215. 403-10 (1990)), Pearson and Lipman's similar searching method (Proc. Natl. Acad. Sci. USA. 85. 2444 (1988)), Smith and Waterman's local homology algorithm (Adv. Appl. Math. 2. 482-9 10 (1981)), and so on have been well known. Further, blastp, one of the BLAST programs provided on the Internet by the National Institutes of 15 Health, has been well known as a means to calculate the homology of two amino acid sequences.

[0020] The lysosomal enzymes are described in detail by taking acid α -glucosidase and α -galactosidase A as an example in detail below.

[0021] Acid α -glucosidase (GAA) is a lysosomal enzyme that has the 20 activity of degrading glycogen. Pompe disease (glycogen storage disease type II) is a disease in which glycogen accumulates in large amounts in the lysosome of cells by genetically lacking most or all of the acid α -glucosidase activity, and the dysfunction of cardiac muscle and skeletal muscle is a main symptom. Pompe disease is classified into 25 infant form, childhood form and adult form. The infant form develops mainly in the early infancy and shows cardiovascular symptoms due to

concentric cardiac hypertrophy, muscle weakness due to skeletal muscle invasion, and hypotonia due to glycogen accumulation in the myocardium, and is characterized by congenital myopathic symptoms. In the childhood form, skeletal muscle symptoms are predominant, and they show motor developmental delay and progressive muscle weakness from the late infancy. Muscle weakness is the main symptom in the adult form.

[0022] α -Galactosidase A (α -GalA) is a lysosomal enzyme that has the activity of hydrolyzing terminal α -galactosyl bonds of glycolipids and glycoproteins. Trihexosylceramide is one of the substrates for α -galactosidase A and undergoes hydrolysis by the enzyme at its terminal hexose moiety. Fabry disease is a disease caused by a genetic loss of most or all of the α -GalA activity. The clinical features of Fabry disease are renal failure, angiokeratoma, and cardiovascular abnormalities, such as ventricular hypertrophy and mitral valve regurgitation. An atypical type of Fabry disease, in which the heart is primarily impaired, could be distinguished, particularly as cardiac Fabry disease.

[0023] As a therapy for the lysosomal disease patient lacking the lysosomal enzyme, the enzyme replacement therapy in which the lysosomal enzyme produced using the gene recombination technology is administered to the patient as a drug is carried out. For example, enzyme replacement therapy using Myozyme (registered trademark), a recombinant human acid α -glucosidase produced by gene recombination technology, is carried out on Pompe disease patients. In addition, enzyme replacement therapy using Fabrazyme or Ripregal

(registered trademarks), a recombinant human α -galactosidase A produced by the gene recombination technology, is carried out for the Fabry disease patient. One of tissues in which lysosomal enzymes used as medicaments in these enzyme replacement therapies should exert their effect is muscle. Thus, by increasing the supply of lysosomal enzymes to the muscle, it may be possible to further ameliorate the muscle dysfunction associated with lysosomal disease.

5 [0024] In the present invention, the term "human acid α -glucosidase" or "hGAA" particularly means a hGAA having an amino acid sequence set forth as SEQ ID NO: 1 which is the same as that of a native hGAA, but the present invention is not limited thereto, and as long as it has hGAA activity, hGAA includes a hGAA to which mutations such as substitutions, deletions, additions, and the like are introduced to the amino acid sequence of a native hGAA. When the amino acids of the 10 amino acid sequence of hGAA are substituted with other amino acids, the number of amino acids to be substituted is preferably from 1 to 10, more preferably from 1 to 5, even more preferably from 1 to 3, and still more preferably from 1 to 2. When the amino acids of the amino acid sequence of a hGAA, the number of amino acids to be deleted is 15 preferably from 1 to 10, more preferably from 1 to 5, even more preferably from 1 to 3, and still more preferably from 1 to 2. A mutation combining such substitution and deletion of amino acids can also be introduced to hGAA. When amino acids are added to a hGAA, preferably 1 to 10, more preferably 1 to 5, even more preferably 1 to 3, and still more preferably 1 to 2 amino acids are added in the amino acid 20 sequence of hGAA or on the N-terminal or C-terminal side. A mutation 25

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combining such addition, substitution and deletion of amino acids can also be introduced to hGAA. The amino acid sequence of the mutated hGAA preferably has 80% or more identity, more preferably 90% or more identity, and even more preferably 95% or more identity to the amino acid sequence of the original hGAA.

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[0025] Here, the term "hGAA has hGAA activity" means that it has not less than 3% activity relative to the activity inherent in the natural type hGAA when conjugated to anti-hTfR antibody. However, the activity is preferably not less than 10%, more preferably 20% or more, even more preferably not less than 50%, and still more preferably not less than 80% relative to the activity inherent in the natural type hGAA. The same is true when hGAA conjugated with the anti-hTfR antibody is mutated.

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[0026] In the present invention, the term "human α -galactosidase A" or " α -GalA" means, in particular, α -GalA having the amino acid sequence set forth as SEQ ID NO: 2 which is the same as that of a native α -GalA, but is not limited thereto, and as long as the α -GalA has the α -GalA activity, the α -GalA also includes mutations such as substitutions, deletions, additions, etc. in the amino acid sequence of the native α -GalA. When amino acids of the amino acid sequence of the α -GalA are substituted with other amino acids, the number of amino acids to be substituted is preferably from 1 to 10, more preferably from 1 to 5, even more preferably from 1 to 3, and still more preferably from 1 to 2. When amino acids in the amino acid sequence of the α -GalA are deleted, the number of amino acids to be deleted is preferably from 1 to 10, more preferably from 1 to 5, even more preferably from 1 to 3, and

still more preferably from 1 to 2. A mutation combining such substitution and deletion of amino acids can also be introduced to the α -GalA. When amino acids are added to the α -GalA, preferably 1 to 10, more preferably 1 to 5, even more preferably 1 to 3, still more preferably 1 to 2 amino acids are added in the amino acid sequence of the α -GalA or on the N-terminal or C-terminal side. A mutation combining such addition, substitution and deletion of amino acids may also be introduced to the α -GalA. The amino acid sequence of the mutated α -GalA preferably has not less than 80% identity, more preferably not less than 90% identity, and even more preferably not less than 95% identity to the amino acid sequence of the original α -GalA.

[0027] Here, when α -GalA has α -GalA activity, it means that it has an not less than 3% activity relative to the activity inherent in the native α -GalA when conjugated with anti-hTfR antibody. However, the activity is preferably not less than 10%, more preferably not less than 20%, even more preferably not less than 50%, and still more preferably not less than 80% relative to the activity inherent in the native α -GalA. The same is true when the α -GalA conjugated with anti-hTfR antibody is mutated.

[0028] In the present invention, the term "antibody" refers primarily to human antibody, mouse antibody, humanized antibody, chimeric antibody of human antibody and other mammalian antibody, and chimeric antibody of mouse antibody and other mammalian antibody, but as long as they have the property of specifically binding to a particular antigen, they are not limited thereto, and there are not any particular limitations to the animal species of the antibody.

[0029] In the present invention, the term "human antibody" refers to an antibody whose entire protein is encoded by a gene originating from human. The term "human antibody", however, also includes an antibody encoded by a gene obtained by introducing a mutation into an original human gene for a purpose of enhancing expression efficacy of the gene, for example, without modifying the original amino acid sequence. The term "human antibody" also includes an antibody in which certain part of the human antibody is replaced with part of another human antibody by combining two or more genes encoding human antibodies. A human antibody includes three complementarity determining regions (CDRs: abbreviation of complementary determining region) in the light chain of the immunoglobulin and three complementarity determining regions (CDRs) in the heavy chain of the immunoglobulin. The three CDRs in the light chain of the immunoglobulin are called, from the N-terminal side, CDR1, CDR2 and CDR3, respectively. The three CDRs in the heavy chain of the immunoglobulin are also called, from the N-terminal side, CDR1, CDR2 and CDR3, respectively. The term "human antibody" also includes a human antibody produced by replacing a CDR of a human antibody with a CDR of another human antibody to modify such properties as the antigen specificity and the affinity of the original human antibodies, etc.

[0030] In the present invention, the term "human antibody" also includes an antibody which is produced through modification of the gene of the original human antibody by introducing a mutation, such as substitution, deletion, addition, to the amino acid sequence of the

original antibody. When replacing one or more amino acids of the amino acid sequence of the original antibody with other amino acids, the number of amino acid replaced may preferably be 1-20, more preferably 1-5, and still more preferably 1-3. When deleting one or 5 more amino acids of the amino acid sequence of the original antibody, the number of amino acids deleted may preferably be 1-20, more preferably 1-5, and still more preferably 1-3. An antibody produced by a combined mutation of these substitution and deletion of amino acids is also a "human antibody". In some cases, one or more amino 10 acids, preferably 1-20, more preferably 1-5, and still more preferably 1-3 amino acids may be added inside the amino acid sequence of the original antibody or on its N- or C-terminal side. An antibody produced by a combined mutation of addition, substitution, and deletion of amino acids is also a "human antibody". The amino acid sequence 15 of such a mutated antibody has a homology of preferably not lower than 80%, more preferably not lower than 90%, still more preferably not lower than 95%, and even more preferably not lower than 98%, to the amino acid sequence of the original antibody. Thus, in the present invention, the term "gene originating from human" includes not only the unmutated gene originating from human but also a gene produced by 20 modifying this.

[0031] The term "mouse antibody" refers to an antibody whose entire protein consists of an amino acid sequence which is the same as an antibody encoded by a gene originating from a mouse. Therefore, the 25 term "mouse antibody" also includes an antibody that is encoded by a gene produced by introducing a mutation into the original mouse gene

without causing a change in its amino acid sequence but in order, for example, to improve the expression efficiency of the gene. Further, the term "mouse antibody" also includes an antibody produced through combining two or more genes encoding mouse antibodies by replacing 5 part of a mouse antibody with part of another mouse antibody. A mouse antibody has three complementarity determining regions (CDRs) in the light chain of the immunoglobulin and three complementarity determining regions (CDRs) in the heavy chain of the immunoglobulin. The three CDRs in the light chain of the immunoglobulin are called, 10 from the N-terminal side, CDR1, CDR2 and CDR3, respectively. The three CDRs in the heavy chain of the immunoglobulin are also called, from the N-terminal side, CDR1, CDR2 and CDR3, respectively. For example, the term "mouse antibody" also includes an antibody produced by replacing a CDR of a mouse antibody with a CDR of another mouse 15 antibody to modify the specificity and affinity of the original mouse antibodies.

[0032] In the present invention, the term "mouse antibody" also includes an antibody which is produced through modification of the gene of the original mouse antibody by introducing a mutation, such as 20 substitution, deletion, addition, to the amino acid sequence of the original antibody. When replacing one or more amino acids of the amino acid sequence of the original antibody with other amino acids, the number of amino acid replaced may preferably be 1-20, more preferably 1-5, and still more preferably 1-3. When deleting one or 25 more amino acids of the amino acid sequence of the original antibody, the number of amino acids deleted may preferably be 1-20, more

preferably 1-5, and still more preferably 1-3. An antibody produced by a combined mutation of these substitution and deletion of amino acids is also included in a "mouse antibody". When adding one or more amino acids, they may be added inside the amino acid sequence of the original antibody or on its N- or C-terminal side, preferably 1-20, more preferably 1-5, and still more preferably 1-3, in number. An antibody produced by a combined mutation of addition, substitution, and deletion of amino acids is also included in a "mouse antibody". The amino acid sequence of such a mutated antibody has a homology of preferably not lower than 80%, more preferably not lower than 90%, still more preferably not lower than 95%, and even more preferably not lower than 98%, to the amino acid sequence of the original antibody. Thus, in the present invention, the term "gene originating from mouse" includes not only the unmutated gene originating from mouse but also a gene produced by modifying this.

[0033] In the present invention, the term "humanized antibody" refers to an antibody in which part of the amino acid sequence of its variable region (e.g., especially the whole or part of its CDRs) originates from a non-human mammal while the rest originates from human. An example of humanized antibody is an antibody produced by replacing the three complementarity determining regions (CDRs) of the light chain of the immunoglobulin and the three complementarity determining regions (CDRs) of the heavy chain of the immunoglobulin constituting a human antibody, with CDRs from a non-human mammal. There is no particular limitation as to the biological species from which those CDRs grafted into a proper position of the human antibody

originate, insofar as it originates from a non-human mammal. Though the species are preferably mouse, rat, rabbit, horse or non-human primate, and more preferably mouse or rat, for example mouse.

5 [0034] In the present invention, the term "chimeric antibody" refers to an antibody produced by connecting fragments of two or more different antibodies originating from two or more different species.

10 [0035] A chimeric antibody comprising a human antibody and a non-human mammalian antibody is an antibody provided by replacing part of a human antibody with part of a non-human mammalian antibody. An antibody is made of an Fc region, a Fab region and a hinge region. A specific example of such chimeric antibodies is a chimeric antibody whose Fc region originates from a human antibody while its Fab region originates from a non-human mammalian antibody. The hinge region either originates from a human antibody or from a 15 non-human mammalian antibody. On the contrary, the term chimeric antibody also includes one whose Fc region originates from a non-human mammalian antibody while its Fab region originates from a human antibody. In such a case also, the hinge region either originates from a human antibody or from a non-human mammalian antibody.

20 [0036] An antibody can be viewed as composed of a variable region and a constant region. Additional examples of chimeric antibodies include an antibody in which the heavy chain constant region (C_H) and the light chain constant region (C_L) both originate from a human antibody while the heavy chain variable region (V_H) and the light chain 25 variable region (V_L) both originate from an antibody of a non-human mammal, and conversely, an antibody in which the heavy chain constant

region (C_H) and the light chain constant region (C_L) both originate from an antibody of a non-human mammal, while the heavy chain variable region (V_H) and the light chain variable region (V_L) both originate from a human antibody. In these, there is no particular limitation as to the biological species of the non-human mammal, insofar as it is a non-human mammal, though the species are preferably mouse, rat, rabbit, horse or non-human primate, more preferably mouse.

5 [0037] A chimeric antibody of a mouse antibody and another mammalian antibody is an antibody in which a part of the mouse antibody is replaced by a part of an antibody of a mammal other than a mouse. An example of such a chimeric antibody includes a chimeric antibody in which the Fc region is derived from a mouse antibody while the Fab region is derived from an antibody of another mammal, and conversely, a chimeric antibody in which the Fc region is derived from 10 another mammal while the Fab region is derived from a mouse antibody. Here, the species of another mammal is not particularly limited as long as it is a mammal other than a mouse, but preferably is a rat, a rabbit, a horse, and a non-human primate.

15 [0038] A chimeric antibody comprising a human antibody and a mouse antibody is designated in particular "human/mouse chimeric antibody". Examples of human/mouse chimeric antibodies include a chimeric antibody in which the Fc region originates from a human antibody while the Fab region originates from a mouse antibody, and conversely, a chimeric antibody whose Fc region originates from mouse antibody, 20 while its Fab region originates from a human antibody. A hinge region either originates from a human antibody or a mouse antibody.

Additional specific examples of human/mouse chimeric antibodies include those whose heavy chain constant region (C_H) and light chain constant region (C_L) originate from a human antibody while its heavy chain variable region (V_H) and light chain variable region (V_L) originate from a mouse antibody, and conversely, those whose heavy chain constant region (C_H) and light chain constant region (C_L) originate from a mouse antibody while its heavy chain variable region (V_H) and light chain variable region (V_L) originate from a human antibody.

[0039] Originally, an antibody is of the basic structure having four polypeptide chains in total, consisting of two immunoglobulin light chains and two immunoglobulin heavy chains. However, in the present invention the term "antibody" refers, besides to an antibody having this basic structure, also to;

(1) an antibody consisting of two polypeptide chains comprising a single immunoglobulin light chain and a single immunoglobulin heavy chain, and, as explained later,

(2) a single-chain antibody consisting of an immunoglobulin light chain which is linked, on the C-terminal side thereof, to a linker sequence which in turn is linked, on the C-terminal side thereof, to an immunoglobulin heavy chain,

(3) single-chain antibodies consisting of an immunoglobulin heavy chain which is linked, on the C-terminal side thereof, to a linker sequence which in turn is linked, on the C-terminal side thereof, to an immunoglobulin light chain, and

(4) one consisting of a Fab region, i.e., a structure left behind by removal of the Fc region from an antibody having the basic structure as

the original meaning, and one consisting of the Fab region and the whole or part of the hinge region (including Fab, F(ab'), and F(ab')₂).

[0040] Here, the term "Fab" refers to a molecule consisting of a single light chain comprising the variable region and the C_L region (light chain constant region) and a single heavy chain comprising the variable region and the C_{H1} region (portion 1 of heavy chain constant region) which are combined by a disulfide bond between their respective cysteine residues.

5 constant region) and a single heavy chain comprising the variable region and the C_{H1} region (portion 1 of heavy chain constant region) which are combined by a disulfide bond between their respective cysteine residues. While the heavy chain in a Fab can include part of the hinge region in addition to the variable region and the C_{H1} region (portion 1 of

10 heavy chain constant region), the hinge region in such a case lacks the cysteine residue that otherwise is present in the hinge region and would serve to link two heavy chains of an antibody together. In Fab, the light

chain and the heavy chain are connected by a disulfide bond formed between the cysteine residue present in the light chain constant region (C_L region) and the cysteine residue located in the heavy chain constant region (C_{H1} region) or the hinge region. As it lacks the cysteine residue in the hinge region which serves to bind two heavy chains of an antibody, Fab consists of a single light chain and a single heavy chain.

15 The light chain of Fab includes a variable region and a C_L region. The heavy chain as a component of Fab may either consist of a variable region and a C_{H1} region or also of part of the hinge region in addition to the variable region and the C_{H1} region. However, in this case, the hinge region is so selected as not to include the cysteine residue that could bind two heavy chains, in order to avoid the formation of a disulfide

20 bond between two heavy chains at their hinge regions. In F(ab'), the heavy chain includes, in addition to a variable region and a C_{H1} region,

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the heavy chain includes, in addition to a variable region and a C_{H1} region,

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the whole or part of a hinge region containing a cysteine residue that could bind two heavy chains. $F(ab')_2$ is a molecule consisting of two $F(ab')$ s bound together through a disulfide bond formed between the cysteine residues present in their respective hinge regions. Further, a 5 polymer such as a dimer and a trimer, which consists of two or more antibodies connected with each other, directly or via a linker, is also included in the term "antibody". Moreover, in addition to the aforementioned, any molecule that includes part of an immunoglobulin molecule and has a property to specifically bind to the antigen is also 10 included in the term "antibody" in the present invention. Thus, in the present invention, the term "immunoglobulin light chain" includes a molecule that is derived from an original immunoglobulin light chain and having the amino acid sequence of the whole or part of its variable region. Likewise, the term "immunoglobulin heavy chain" includes a 15 molecule that is derived from an original immunoglobulin heavy chain and having the amino acid sequence of the whole or part of its variable region. Therefore, insofar as having the whole or part of the amino acid sequence of the variable region, a molecule is included in the term "immunoglobulin heavy chain", even if it lacks its Fc region, for 20 example.

[0041] Also, in the above, the Fc or F_c region refers to a region including fragments consisting of a C_{H2} region (portion 2 of the constant region of the heavy chain) and a C_{H3} region (portion 3 of the constant region of the heavy chain) in the antibody molecule.

25 [0042] Furthermore, in the present invention, the term "antibody" also includes:

(5) scFab, scF(ab'), and scF(ab')₂, which are single-chain antibodies produced by binding the light chain to the heavy chain that form, respectively, the Fab, F(ab') and F(ab')₂ mentioned in (4) above, via a linker sequence. Such scFab, scF(ab') and scF(ab')₂ may be a molecule in which either the light chain is linked, on the C-terminal side thereof, to a linker sequence, which in turn is linked, on the C-terminal side thereof, to the heavy chain, or the heavy chain is linked, on the C-terminal side thereof, to a linker sequence, which in turn is linked, on the C-terminal side thereof, to the light chain. Furthermore, a scFv, which is a single-chain antibody provided by binding the light chain variable region to the heavy chain variable region, via a linker sequence between them, is also included in the term "antibody" in the present invention. Such scFv may be a molecule in which either the light chain variable region is linked, on the C-terminal side thereof, to a linker sequence, which in turn is linked, on the C-terminal side thereof, to the heavy chain variable region, or the heavy chain variable region is linked, on the C-terminal side thereof, to a linker sequence, which in turn is linked, on the C-terminal side thereof, to the light chain variable region.

[0043] In the present invention, the term "single-chain antibody" refers to a protein in which an amino acid sequence comprising the whole or part of an immunoglobulin light chain variable region linked, on the C-terminal side thereof, to a linker sequence, which in turn is linked, on the C-terminal side thereof, to the amino acid sequence of the whole or part of an immunoglobulin heavy chain variable region, and having an ability to specifically bind a certain antigen. For example, those

shown in (2), (3), and (5) above are included in the single-chain antibody. Further, a protein in which an amino acid sequence comprising the whole or part of an immunoglobulin heavy chain variable region is linked, on the C-terminal side thereof, to a linker sequence, which in turn is further linked, on the C-terminal side thereof, to the amino acid sequence of the whole or part of an immunoglobulin light chain variable region, and which has an ability to specifically bind to a certain antigen, is also included in the term "single-chain antibody" in the present invention. In a single-chain antibody in which an immunoglobulin heavy chain is linked, on the C-terminal side thereof and via a linker sequence, to an immunoglobulin light chain, the immunoglobulin heavy chain generally lacks the Fc region. An immunoglobulin light chain variable region has three complementarity determining regions (CDRs) which participate in determining the antigen specificity of an antibody. Likewise, an immunoglobulin heavy chain variable region also has three CDRs. Those CDRs are the primary regions that determine the antigen specificity of an antibody. Therefore, a single-chain antibody preferably contains all the three CDRs of the immunoglobulin heavy chain and all the three CDRs of the immunoglobulin light chain. However, it is also possible to provide a single-chain antibody in which one or more of those CDRs are deleted, insofar as the antigen-specific affinity of the antibody is retained.

[0044] In a single-chain antibody, the linker sequence placed between the light chain and the heavy chain of the immunoglobulin is a peptide chain consisting of preferably 2 to 50, more preferably 8 to 50, still more preferably 10 to 30, even more preferably 12 to 18, or 15 to 25, for

example 15 or 25 amino acid residues. While there is no particular limitation as to the specific amino acid sequence of such a linker sequence insofar as the anti-hTfR antibody comprising the both chains linked thereby retains the affinity to hTfR, it is preferably made of 5 glycine only, or of glycine and serine. For example, there are the amino acid sequence Gly-Ser, the amino acid sequence Gly-Gly-Ser, the amino acid sequence Gly-Gly-Gly, the amino acid sequence Gly-Gly-Gly-Gly-Ser (SEQ ID NO:3), the amino acid sequence Gly-Gly-Gly-Gly-Gly-Ser (SEQ ID NO:4), the amino acid sequence Gly-Gly-Gly-Gly-Gly (SEQ ID NO:21), or a sequence which includes a 10 sequence corresponding to 2 to 10 or 2 to 5 of any of those amino acid sequences consecutively linked. For example, in linking the amino acid sequence of the entire immunoglobulin heavy chain variable region on the C-terminal side thereof and via a linker sequence, to 15 immunoglobulin light chain variable region, a preferable linker sequence is a linker sequence consisting of a total of 15 amino acids corresponding to three of the amino acid sequence Gly-Gly-Gly-Gly-Ser (SEQ ID NO:3) consecutively linked.

[0045] In the present invention, the term "human transferrin receptor" 20 or "hTfR" refers to a membrane protein having the amino acid sequence set forth as SEQ ID NO:5. The anti-hTfR antibody of the present invention is, in one of its embodiments, that which binds to the region from the cysteine residue at the position 89th from the N-terminus to the phenylalanine at the C-terminus in the amino acid sequence set forth as 25 SEQ ID NO:5 (the extracellular region of hTfR), though it is not limited to this embodiment. Further in the present invention, the term "monkey

transferrin receptor" or "monkey TfR" refers to, in particular, a membrane protein having the amino acid sequence set forth as SEQ ID NO: 6 derived from a crab-eating macaque (*Macaca fascicularis*). The anti-hTfR antibody of one embodiment of the present invention, also binds to the region from the cysteine residue at the position 89th from the N-terminus to the phenylalanine at the C-terminus in the amino acid sequence set forth as SEQ ID NO:6 (the extracellular region of monkey TfR).

[0046] As a method for producing antibody against hTfR, it is common practice to generate recombinant human transferrin receptor (rhTfR) using cells transduced with expression vectors incorporating hTfR genes and immunize an animal such as a mouse with this rhTfR. Hybridoma cells capable of producing the antibody can be produced by collecting antibody-producing cells against hTfR from the animal after immunization and fusing the cells with myeloma cells.

[0047] Further, cells producing an antibody to hTfR can also be obtained by collecting immunocompetent cells from an animal such as mouse, and immunizing them with rhTfR by in vitro immunization. In conducting immunization by in vitro immunization, there is no particular limitation as to the animal species from which the immunocompetent cells are derived, though preferred are mouse, rat, rabbit, guinea pig, dog, cat, horse, and primates including human, and more preferred are mouse, rat and human, and still more preferably mouse and human. As mouse immunocompetent cells, spleen cells prepared from mouse spleen may be used, for example. As human immunocompetent cells, such cells can be used as prepared from human

peripheral blood, bone marrow, spleen, and the like. By immunizing human immunocompetent cells according to in vitro immunization, a human antibody to hTfR can be obtained.

[0048] After immunizing the immune system cells by in vitro immunization, the cells are fused with myeloma cells, whereby hybridoma cells capable of producing the antibody can be produced. In addition, it is also possible to extract mRNA from the cells after immunization to synthesize cDNA, amplify DNA fragments containing genes encoding the light and heavy chain of immunoglobulin by 5 PCR-reaction using this cDNA as a template, and artificially reconstruct antibody genes using the amplified DNA fragments.

[0049] Hybridoma cells as obtained by the above method also include cells producing antibodies that recognize proteins other than hTfR as antigens. Also, all of the hybridoma cells producing the anti-hTfR 10 antibody do not necessarily produce an anti-hTfR antibody that exhibits the desired affinity for hTfR.

[0050] Likewise, artificially reconstructed antibody genes include such genes as encode antibodies recognizing other proteins than hTfR as antigens. Moreover, not all the genes encoding anti-hTfR antibodies 15 necessarily have desired properties such as encoding an anti-hTfR antibody exhibiting high affinity to hTfR.

[0051] Therefore, a selection step is necessary to select hybridoma cells producing an antibody having desired properties (such as high affinity to hTfR) from the hybridoma cells freshly obtained above. Further, in 20 the case where antibody genes are artificially reconstructed, a selection step is necessary to select from the antibody genes a gene encoding an

antibody having desired properties (such as high affinities to hTfR).

[0052] For example, for selecting hybridoma cells which produce high affinity antibodies to anti-hTfR antibody, a method is employed in which recombinant hTfR is added to a plate and held by it, then the culture supernatant of the hybridoma cells is added, and after removing antibody unbound to the recombinant hTfR from the plate, the amount of the antibody held by the plate is measured. According to this method, the higher the affinity to hTfR of the antibody contained in the culture supernatant of the hybridoma cells added to the plate is, the greater the amount of antibody held by the plate becomes. Therefore, by measuring the amount of the antibody held by the plate, it is possible to select those hybridoma cells corresponding to the plates where the antibody is held in the greater amount as cell lines producing an anti-hTfR antibody having the relatively higher affinity to hTfR. It is also possible to isolate the gene encoding the high-affinity antibody by extracting mRNAs from each cell line selected in this manner, synthesizing cDNAs, and amplifying a DNA fragment containing the gene encoding the anti-hTfR antibody by PCR using the cDNA as a template.

[0053] In order to select the gene encoding the high-affinity anti-hTfR antibody from the above artificially reconstructed antibody genes, the artificially reconstructed antibody genes are once incorporated into an expression vector, and the expression vector then is introduced into host cells. Although there is no particular limitation as to the cells to be employed as host cells, even whether they are prokaryotic eukaryotic, insofar as they can express the antibody gene after introduction of an

expression vector having the incorporated artificially reconstructed antibody gene, preferred are cells originating mammals such as human, mouse, Chinese hamster, and the like, and particularly preferred are CHO cells originating from Chinese hamster ovary cells, or NS/0 cells originating from mouse myeloma. Further, there is no particular limitation as to an expression vector to be employed for incorporation of the antibody encoding gene and expression of it, and any expression vector may be used as far as it can express the gene when incorporated into mammalian cells. The gene incorporated into an expression vector is located downstream of a DNA sequence that can regulate the frequency of transcription of a gene in mammalian cells (gene expression regulatory site). Examples of gene expression regulatory sites that may be employed in the present invention include cytomegalovirus-derived promoter, SV40 early promoter, human elongation factor-1 α (EF-1 α) promoter, human ubiquitin C promoter.

[0054] Mammalian cells having such an introduced expression vector come to express the antibody encoded by artificially reconstructed gene above incorporated in the expression vector. In order to select those cells which produce a high-affinity antibody to anti-hTfR antibody from the above obtained cells expressing the artificially reconstructed antibody, a method is employed in which the recombinant hTfR is added to a plate and held by it, then the recombinant hTfR is contacted by the culture supernatant of the cells, and after the removal of antibody unbound to the recombinant hTfR from the plate, the amount of the antibody held by the plate is measured. According to this method, the higher the affinity to hTfR of the antibody contained in the cells culture

supernatant is, the greater the amount of antibody held by the plate becomes. Therefore, by measuring the amount of the antibody held by the plate, one can select those cells corresponding to the plate where the antibody is held in the greater amount, as a cell line producing an anti-hTfR antibody having relatively the high-affinity anti-hTfR antibody, and eventually can select a gene encoding an anti-hTfR antibody having a high-affinity anti-hTfR antibody to hTfR. Using cell line selected in this manner, one can perform PCR to amplify a DNA fragment containing the gene encoding the anti-hTfR antibody to isolate the gene encoding the high-affinity antibody.

10 [0055] Selection of the gene encoding a high affinity anti-hTfR antibody from the above artificially reconstructed antibody genes can also be carried out by incorporating the artificially reconstructed antibody genes into an expression vector, introducing the expression vector into *E. coli* cells, culturing the *E. coli* cells, and selecting the *E. coli* cells having the desired gene, in the same manner as in the above selection of hybridoma cells, using the culture supernatant of the *E. coli* cells or an antibody-containing solution prepared by lysing the *E. coli* cells. *E. coli* cells thus selected express the gene encoding an anti-hTfR antibody having a relatively high affinity to hTfR. From this cell line, the gene encoding the anti-hTfR antibody having a relatively the high-affinity anti-hTfR antibody to hTfR can be isolated. In order to allow the antibody to be secreted into the *E. coli* culture supernatant, the antibody gene may be incorporated into the expression vector so that a secretion signal sequence is attached on the N-terminal side of the gene.

[0056] Another method for selection of the gene encoding a high-affinity anti-hTfR antibody is a method in which the antibody encoded by the above artificially reconstructed antibody gene is expressed and retained on phage particles. For this, the antibody gene is reconstructed as a gene encoding a single-chain antibody. A method for retaining phage particles to retain an antibody on their surface is disclosed in international publications WO1997/09436 and WO1995/11317, and the like, and thus well known. In order to select phages retaining the high-affinity antibody to anti-hTfR antibody from the phages retaining the antibodies encoded by the artificially reconstructed antibody genes, a method is employed in which a recombinant hTfR from the plate is added to a plate and held by, contacted by the phages, and after removal of the phages unbound to the recombinant hTfR, the amount of the phages held by the plate is measured. According to this method, the higher the affinity to hTfR of the antibody retained on the phage particles is, the greater the amount of the phage held by the plate becomes. Therefore, by measuring the amount of the phage held by the plate, one can select the phage particles corresponding to the plate where the phages' were held in the greater amount, as the phage particles producing anti-hTfR antibody having a relatively the high-affinity anti-hTfR antibody to hTfR, and eventually can select the gene encoding the high-affinity anti-hTfR antibody to hTfR. Using the phage particles thus selected, PCR can be performed to amplify a DNA fragment containing the gene encoding the anti-hTfR antibody and isolate the gene encoding the high-affinity antibody.

[0057] It is possible to prepare cDNA or phage DNA from the above

cells such as the hybridoma cells producing the high-affinity antibody to anti-hTfR, or from the above phage particles retaining high-affinity antibody to anti-hTfR, and perform PCR or the like using it as a template to amplify and isolate a DNA fragment containing the gene encoding the whole or part of the anti-hTfR antibody light chain, the anti-hTfR antibody heavy chain, or a single-chain antibody, as an anti-hTfR antibody. In the same manner, it is also possible to perform PCR or the like to amplify and isolate a DNA fragment containing the gene encoding the whole or part of the light chain variable region of the anti-hTfR antibody, or a DNA fragment containing the gene encoding the whole or part of the heavy chain variable region of the anti-hTfR antibody.

[0058] A high-affinity anti-hTfR antibody can be obtained by incorporating the whole or part of the gene encoding the light chain and the heavy chain of this high-affinity anti-hTfR antibody into an expression vector, transforming host cells such as mammalian cells with this expression vector, and culturing the obtained transformant cells. Using the nucleotide sequence of the isolated gene encoding the anti-hTfR antibody, it is also possible to translate the amino acid sequence of the anti-hTfR antibody, and artificially synthesize a DNA fragment encoding the same amino acid sequence. In artificially synthesizing a DNA fragment, the expression level of the anti-hTfR antibody in the host cells can be enhanced by proper selection of the codons.

[0059] In order to introduce a mutation such as substitution, deletion, addition and the like into the amino acid sequence of the original

anti-hTfR antibody, a mutation may be introduced as desired into the gene encoding the anti-hTfR antibody contained in the isolated DNA fragment. Though the gene encoding the mutated anti-hTfR antibody has a homology preferably not lower than 80%, more preferably not lower than 90%, to the original gene, there is no particular limitation as to the level of homology as far as the affinity for hTfR is not lost. By introducing a mutation into the amino acid sequence so as to modify the number or the type of sugar chains bound to the anti-hTfR antibody, it is also possible to enhance the stability of the anti-hTfR antibody in the body.

[0060] When introducing a mutation into the gene encoding the whole or part of the light chain variable region of the anti-hTfR antibody, the gene thus mutated has a homology that is preferably not lower than 80%, more preferably not lower than 90%, to the original gene, though there is no particular limitation as to the level of homology as far as the affinity for hTfR is not lost. When replacing one or more amino acids of the amino acid sequence of the light chain variable region with other amino acids, the number of amino acids to be replaced is preferably 1 to 10, more preferably 1 to 5, still more preferably 1 to 3, and even more preferably 1 or 2. When deleting one or more amino acids of the amino acid sequence of the light chain variable region, the number of amino acid to be deleted is preferably 1 to 10, more preferably 1 to 5, still more preferably 1 to 3, even more preferably 1 or 2. A combined mutation of these substitution and deletion of amino acids can also be carried out. When adding one or more amino acids to the light chain variable region, they may be added inside, or on the N-terminal side or

C-terminal side of, the amino acid sequence of the light chain variable region, and the number of amino acids added is preferably 1 to 10, more preferably 1 to 5, still more preferably 1 to 3, and even more preferably 1 or 2. A combined mutation of these addition, substitution, and deletion of amino acids can also be carried out. The amino acid sequence of the light chain variable region thus mutated has a homology that is preferably not lower than 80%, more preferably not lower than 90%, still more preferably not lower than 95% to the amino acid sequence of the original light chain variable region. In particular, when replacing one or more amino acids of the amino acid sequence of CDR with other amino acids, the number of amino acid replaced is preferably 1 to 5, more preferable 1 to 3, still more preferably 1 or 2. When deleting one or more amino acid of the amino acid sequence of CDR, the number of amino acids to be deleted is preferably 1 to 5, more preferably 1 to 3, still more preferably 1 or 2. A combined mutation of these substitution and deletion of the amino acid can also be carried out. When adding one or more amino acids, they may be added inside, or on the N-terminal side or C-terminal side of, the amino acid sequence, and the number of amino acids added is preferably 1 to 5, more preferably 1 to 3, still more preferably 1 or 2. A combined mutation of these addition, substitution, and deletion of amino acids can also be carried out. The amino acid sequence of respective mutated CDR has a homology that is preferably not lower than 80%, more preferably not lower than 90%, and still more preferably not lower than 95% to the amino acid sequence of the original CDR.

[0061] When introducing mutation into the gene encoding the whole or

part of the heavy chain variable region of the anti-hTfR antibody, the gene thus mutated has a homology that is preferably not lower than 80%, more preferably not lower than 90%, to the original gene, though there is no particular limitation as to the level of homology as far as the 5 affinity for hTfR is not lost. When replacing one or more amino acids of the amino acid sequence of the heavy chain variable region with other amino acids, the number of amino acids to be replaced is preferably 1 to 10, more preferably 1 to 5, still more preferably 1 to 3, and even more preferably 1 or 2. When deleting one or more amino 10 acids of the amino acid sequence of the heavy chain variable region, the number of amino acids to be deleted is preferably 1 to 10, more preferably 1 to 5, still more preferably 1 to 3, even more preferably 1 or 2. A combined mutation of these substitution and deletion of amino acids can also be carried out. When adding one or more amino acid to 15 the heavy chain variable region, they may be added inside, or on the N-terminal side or C-terminal side of, the amino acid sequence of the heavy chain variable region, and the number of amino acids added is preferably 1 to 10, more preferably 1 to 5, still more preferably 1 to 3, and even more preferably 1 or 2. A combined mutation of these addition, substitution, and deletion of amino acids can also be carried 20 out. The amino acid sequence of the heavy chain variable region thus mutated has a homology that is preferably not lower than 80%, more preferably not lower than 90%, still more preferably not lower than 95% to the amino acid sequence of the original heavy chain variable region. 25 In particular, when replacing one or more amino acids of the amino acid sequence of CDR with other amino acids, the number of amino acid

replaced is preferably 1 to 5, more preferably 1 to 3, still more preferably 1 or 2. When deleting one or more amino acid of the amino acid sequence of CDR, the number of amino acids to be deleted is preferably 1 to 5, more preferably 1 to 3, still more preferably 1 or 2.

5 A combined mutation of these substitution and deletion of the amino acid can also be carried out. When adding one or more amino acids, they may be added inside, or on the N-terminal side or C-terminal side of, the amino acid sequence, and the number of amino acids added is preferably 1 to 5, more preferably 1 to 3, still more preferably 1 or 2.

10 A combined mutation of these addition, substitution, and deletion of amino acids can also be carried out. The amino acid sequence of respective mutated CDR has a homology that is preferably not lower than 80%, more preferably not lower than 90%, and still more preferably not lower than 95% to the amino acid sequence of the

15 original CDR.

[0062] A mutation may be introduced into both the variable regions of the light chain and the heavy chain of the anti-hTfR antibody, by combining the above mutation into the light chain variable region of the anti-hTfR antibody and the above mutation into the heavy chain variable region of the anti-hTfR antibody.

[0063] Substitutions of amino acids in the amino acid sequences of the light and heavy chain of the above anti-hTfR antibody to other amino acids include a substitution between amino acids classified into the same group, such as aromatic amino acids (Phe, Trp, Tyr), aliphatic amino acids (Gly, Ala, Leu, Ile, Val), amide-type amino acids (Gln, Asn), basic amino acids (Lys, Arg, His), acidic amino acids (Glu, Asp),

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amino acids with hydroxyl groups (Ser, Thr), branched amino acids (Val, Leu, Ile), amino acids with smaller side chains (Gly, Ala, Ser, Thr, Met), amino acids with nonpolar side chains (Ala, Val, Leu, Ile, Pro, Phe, Met). It is expected that the substitution between such analogous amino acids does not bring about any change to the phenotype of the protein (i.e., it is conservative amino acid substitution). Examples of conservative amino acid substitutions are well known in the art and described in various references (see, e.g., Bowie et al., *Science*, 247:1306-1310 (1990)).

5 10 [0064] When amino acids are added to the C-terminal or N-terminal end of an anti-hTfR antibody by mutation and the anti-hTfR antibody and a drug (including a physiologically active protein) to exert its function in muscle are linked via the amino acids, the amino acids shall constitute a part of the anti-hTfR antibody in the conjugate.

15 20 [0065] The anti-hTfR antibody obtained by culturing the cells selected by the above methods and the like, as producing an anti-hTfR antibody that has a relatively the high-affinity anti-hTfR antibody to hTfR, and the anti-hTfR antibody obtained by expression of the gene encoding a high-affinity anti-hTfR antibody, may be modified by introducing a mutation into their amino acid sequences, such as substitution, deletion, addition to give them desired properties. Introduction of a mutation into the amino acid sequence of the anti-hTfR antibody may be performed by introducing a mutation into the gene corresponding to the amino acid sequence.

25 [0066] The affinity of an anti-hTfR antibody to hTfR can be adjusted as desired by introduction of a mutation, such as substitution, deletion, and

addition, into the amino acid sequence of a variable region of the antibody. For example, if an antibody has such a high affinity to its antigen that leads to too low a dissociation constant in water, there is a possibility that the antibody could, after administered to the body, fail to dissociate from the antigen, thereby leading to a functional disadvantage. In such a case, a most preferable antibody suitable to a given purpose can be obtained by introducing a mutation into the variable region of the antibody so as to adjust its dissociation constant stepwise to 2 to 5 times, 5 to 10 times, 10 to 100 times, and so on, that of the original antibody. Conversely, the dissociation constant can be adjusted stepwise to 1/2 to 1/5 times, 1/5 to 1/10 times, 1/10 to 1/100 times, and so on, that of the original antibody, by introducing a mutation.

[0067] Introduction of a mutation such as substitution, deletion and addition to the amino acid sequence of the anti-hTfR antibody can be performed by introducing a mutation into certain positions of the nucleotide sequence of the gene either, for example, by PCR or the like using the gene encoding the anti-hTfR antibody as a template, or by random introduction of a mutation.

[0068] Introduction of a mutation into the amino acid sequence of the anti-hTfR antibody for adjusting the affinity of the antibody to hTfR can be carried out by, for example, incorporating a gene encoding the anti-hTfR antibody as a single-chain antibody into a phagemid, preparing with this phagemid a phage with expressed single-chain antibody on the surface of its capsid, letting the phage multiply while introducing a mutation into the gene encoding the single-chain antibody

by application of a mutagen or the like, and selecting, from the multiplied phage, a phage expressing a single-chain antibody having a desired dissociation constant either by the method described above or by purification using an antigen column under a certain condition.

5 [0069] If an antibody having a relatively high-affinity to hTfR and obtained by the above method in which those cells producing a high affinity antibody were selected, is an antibody of a non-human animal, it may be converted to a humanized antibody. A humanized antibody is an antibody produced by using an amino acid sequence of part of the
10 variable region (e.g., the whole or part of the CDRs) of a non-human animal antibody, and replacing a proper region of a human antibody with the sequence (implantation) while maintaining the specificity to the antigen. Examples of humanized antibodies include an antibody produced by replacing the three complementarity determining regions
15 (CDRs) in the immunoglobulin light chain and the three complementarity determining regions (CDRs) in the immunoglobulin heavy chain, both constituting a human antibody, with CDRs of a non-human mammal. Though there is no particular limitation as to the biological species from which the CDRs to be incorporated into the
20 human antibody are derived so long as it is a non-human mammal, it preferably is a mouse, rat, rabbit, horse, and non-human primate, more preferably a mouse and rat, and still more preferably a mouse. Here, it is well known that it may be necessary to reproduce the original activity of the donor antibody by replacing the corresponding part of the human
25 antibody as the acceptor by the amino acid sequence of the region outside the CDR which is involved in the structure retention of the CDR

or the binding with the antigen, as well as the CDR of the antibody of the non-human mammal. The region outside the CDR is also referred to as a framework region (FR). Thus, the production of the humanized antibody involves transplanting CDRs (and optionally surrounding FRs) of the non-human mammalian antibody in place of the CDRs (and optionally surrounding FRs) of the variable regions of the human antibody.

[0070] As aforementioned, in a humanized antibody, the regions of a non-human mammalian animal antibody to be implanted into the variable regions of the original human antibody generally include CDRs themselves, or CDRs and their neighboring part of FRs. However, such FRs implanted together with CDRs also play a role either in maintaining the structure of the CDRs or in binding to the antigen, thus having a substantial function in determining the complementarity of an antibody, and the term "CDR" in the present invention, therefore, refers to such regions that are, or could be, taken from a non-human mammalian animal antibody and implanted into a humanized antibody, in preparing a humanized antibody. Thus, a region generally considered to be in a FR region is included in a CDR in the present invention as far as it takes part either in maintaining the structure of the CDR or in binding to the antigen, and is thus considered to have a substantial function in determining the complementarity of the antigen.

[0071] A detailed explanation will be given below regarding the case where the anti-hTfR antibody is a humanized antibody or human antibody. In human antibody light chain, there are λ and κ chains. The light chain constituting the human antibody may either be λ and κ

chain. And in human heavy chain, there are γ , μ , α , σ , and ε chains, which correspond to IgG, IgM, IgA, IgD and IgE, respectively. Though the heavy chain constituting the anti-hTfR antibody may be any of γ , μ , α , σ , and ε chains, preferred is a γ chain. Further, in γ chain of human heavy chain, there are γ_1 , γ_2 , γ_3 and γ_4 chains, which correspond to IgG1, IgG2, IgG3 and IgG4, respectively. Where the heavy chain constituting the anti-hTfR antibody is a γ chain, though the γ chain may be any of γ_1 , γ_2 , γ_3 and γ_4 chains, preferred is a γ_1 or γ_4 chain. In the case where the anti-hTfR antibody is a humanized antibody or human antibody and IgG, the human antibody light chain may either be λ chain or κ chain, and though the human antibody heavy chain may either be γ_1 , γ_2 , γ_3 and γ_4 chains, preferred is a γ_1 or γ_4 chain. For example, a preferable embodiment of anti-hTfR antibody includes one whose light chain is a λ chain and heavy chain is a γ_4 chain, and one whose light chain is a λ chain and heavy chain is a γ_1 .

[0072] In the present invention, the pharmaceutical agent to be conjugated to the anti-hTfR antibody, including a biologically active protein, is the agent to exert its function in muscle. Methods of conjugating the anti-hTfR antibody to such an agent include conjugation via a non-peptide linker or a peptide linker. As the non-peptide linker, polyethylene glycol, polypropylene glycol, copolymer of ethylene glycol and propylene glycol, polyoxyethylated polyol, polyvinyl alcohol, polysaccharide, dextran, polyvinyl ether, biodegradable polymer, lipid polymer, chitin, and hyaluronic acid, a derivative thereof, or a combination thereof can be used. A peptide linker is a peptide chain composed of peptide-bonded 1 to 50 amino acids, or a derivative

thereof, wherein the N-terminal and C-terminal ends of the peptide linker form covalent bonds with either an anti-hTfR antibody or the agent, respectively, thereby linking the anti-hTfR antibody and the agent.

5 [0073] The conjugate of an anti-hTfR antibody in the present invention and a pharmaceutical agent using PEG as a non-peptide linker is particularly referred to as an anti-hTfR antibody-PEG-agent. The anti-hTfR antibody-PEG-agent can be produced by conjugating anti-hTfR antibody and PEG to produce anti-hTfR antibody-PEG, followed by conjugation of anti-hTfR antibody-PEG with the agent. Alternatively, the anti-hTfR antibody-PEG-agent can be produced by conjugating a pharmaceutical agent with PEG to produce an agent-PEG, and then conjugating the agent-PEG with an anti-hTfR antibody. PEG modified with a functional group such as carbonate, carbonylimidazole, active ester of carboxylic acid, azlactone, cyclic imidothion, isocyanate, isothiocyanate, imidate, or aldehyde is used to conjugate PEG to anti-hTfR antibody and the agent. The functional group introduced into the PEG reacts mainly with the anti-hTfR antibody and the amino group in the agent molecule, whereby the PEG and hTfR antibody and the agent are covalently bound. There is no particular limitation on the molecular weight and shape of the PEG used at this time, but the average molecular weight (MW) thereof is preferably from MW=500 to 60000, more preferably from MW=500 to 20000. For example, the PEG having an average molecular weight of about 300, about 500, about 1000, about 2000, about 4000, about 10000, about 20000, or the like can be suitably used as a non-peptide linker.

[0074] For example, "anti-hTfR antibody-PEG" can be prepared by mixing the anti-hTfR antibody with a polyethylene glycol having aldehyde groups as functional groups (ALD-PEG-ALD) so that the molar ratio of ALD-PEG-ALD to the antibody is 11, 12.5, 15, 110, 120 and the like, and then adding to the mixture a reducing agent such as NaCNBH₃ to let a reaction take place. The anti-hTfR antibody-PEG is then reacted with a pharmaceutical agent in the presence of a reducing agent, such as NaCNBH₃, to obtain an anti-hTfR antibody-PEG-agent. Conversely, an anti-hTfR antibody-PEG-agent can also be obtained by first conjugating the agent with ALD-PEG-ALD to produce an agent-PEG, and then conjugating the agent-PEG with an anti-hTfR antibody.

[0075] When the pharmaceutical agent is a biologically active protein (target protein), the anti-hTfR antibody and the target protein can be conjugated to the C-terminal side or the N-terminal side of the heavy or light chain of the anti-hTfR antibody via a linker sequence or directly, respectively, to the N-terminal side or the C-terminal side of the target protein by a peptide bond. The fusion protein obtained by conjugating the anti-hTfR antibody and the target protein in this manner can be obtained by integrating a DNA fragment in which a cDNA encoding the target protein is placed in frame into an expression vector for a mammalian cell in series or by interposing a DNA sequence encoding a linker sequence on the 3' end side or the 5' end side of a cDNA encoding a heavy or light chain of an anti-hTfR antibody, and culturing a mammalian cell into which the expression vector has been introduced. In this mammalian cell, when a DNA fragment encoding a target protein

is linked to a heavy chain, an expression vector for a mammalian cell incorporating a cDNA fragment encoding a light chain of an anti-hTfR antibody is also introduced into the same host cell, and when a DNA fragment encoding a target protein is linked to a light chain, an expression vector for a mammalian cell incorporating a cDNA fragment encoding a heavy chain of an anti-hTfR antibody is also introduced into the same host cell. When the anti-hTfR antibody is a single-chain antibody, a fusion protein in which an anti-hTfR antibody and a target protein are conjugated can be obtained by integrating a DNA fragment, 5 in which a cDNA encoding a single-chain anti-hTfR antibody is linked on the 5' end side or the 3' end side of a cDNA encoding the target protein directly or with a DNA sequence encoding a linker sequence interposed therebetween, into an expression vector (for an eukaryotic 10 cell such as a mammalian cell, a yeast, or a prokaryotic cell such as *E. coli*), and expressing the fusion protein in the cell into which the expression vector is introduced. The fusion protein is to be understood 15 as one form of a conjugate.

[0076] In a fusion protein of the type in which a pharmaceutical agent is linked to the anti-hTfR antibody light chain on the C-terminal side thereof, the anti-human transferrin receptor antibody comprises an amino acid sequence including the whole or part of the light chain variable region and an amino acid sequence including the whole or part of the heavy chain variable region, and the agent is linked to the light chain of this anti-human transferrin receptor antibody on the C-terminal 20 side thereof. Here, the anti-hTfR antibody light chain and the agent 25 may be linked together, directly or via a linker.

[0077] In a fusion protein of the type in which a pharmaceutical agent is linked to the anti-hTfR antibody heavy chain on the C-terminal side thereof, the anti-human transferrin receptor antibody comprises an amino acid sequence including the whole or part of the light chain variable region and an amino acid sequence including the whole or part of the heavy chain variable region, and the agent is linked to the heavy chain of this anti-human transferrin receptor antibody on the C-terminal side thereof. Here, the anti-hTfR antibody heavy chain and the agent may be linked together, directly or via a linker.

[0078] In a fusion protein of the type in which a pharmaceutical agent is linked to the anti-hTfR antibody light chain on the N-terminal side thereof, the anti-human transferrin receptor antibody comprises an amino acid sequence including the whole or part of the light chain variable region and an amino acid sequence including the whole or part of the heavy chain variable region, and the agent is linked to the light chain of this anti-human transferrin receptor antibody on the N-terminal side thereof. Here, the anti-hTfR antibody light chain and the agent may be linked together, directly or via a linker.

[0079] In a fusion protein of the type in which a pharmaceutical agent is linked to the anti-hTfR antibody heavy chain on the N-terminal side thereof, the anti-human transferrin receptor antibody comprises an amino acid sequence including the whole or part of the light chain variable region and an amino acid sequence including the whole or part of the heavy chain variable region, and the agent is linked to the heavy chain of this anti-human transferrin receptor antibody on the N-terminal side thereof. Here, the anti-hTfR antibody heavy chain and the agent

may be linked together, directly or via a linker.

[0080] In the above, the linker sequence placed between the anti-hTfR antibody and the agent may be a peptide chain consisting preferably of 1 to 50, more preferably of 1 to 17, still more preferably of 1 to 10, even more preferably of 1 to 5 amino acids, and in accordance with the agent to be linked to the anti-hTfR antibody, the number of amino acids of the linker sequence may be adjusted to 1, 2, 3, 1-17, 1-10, 10-40, 20-34, 23-31, 25-29, etc., as desired. Though there is no particular limitation as to amino acid sequence of the linker sequence insofar as the anti-hTfR antibody linked by it retains the affinity to hTfR and the agent linked by the linker sequence also exhibit the protein's own physiological activity under a physiological condition, the linker may preferably be composed of glycine and serine. Such linker sequences are preferably, but not limited to, those composed of glycine and serine, for example, amino acid sequence Gly-Ser, amino acid sequence Gly-Gly-Ser, amino acid sequence Gly-Gly-Gly-Gly-Ser (SEQ ID NO: 3), amino acid sequence Gly-Gly-Gly-Gly-Gly-Ser (SEQ ID NO: 4), or a sequence composed of 1 to 50 amino acids in which these amino acid sequences are repeated for 1 to 10 or 2 to 5 times, or a sequence in which these amino acid sequences are repeated for 2 to 17, 2 to 10, 10 to 40, 20 to 34, 23 to 31, or 25 to 29 times, etc., so long as the linked anti-hTfR antibody retain affinity for hTfR and the target protein linked by the linker sequences can exert physiological activity of the protein under physiological conditions. For example, that having the amino acid sequence Gly-Ser can be suitably used as a linker sequence.

[0081] In the case where the anti-hTfR antibody is a humanized

antibody or a human antibody, the anti-hTfR antibody and a target protein may be bound together by linking the anti-hTfR antibody, at the N-terminus (or the C-terminus) of the heavy chain or light chain, via a linker sequence or directly, to the C-terminus (or the N-terminus), respectively, of the target protein, by peptide bonds. When linking the target protein to the anti-hTfR antibody heavy chain on the N-terminal side (or to the C-terminal side) thereof, the C-terminus (or the N-terminus), respectively, of the target protein is linked to the N-terminus (or the C-terminus) of the γ , μ , α , σ or ε chain of anti-hTfR antibody, via a linker sequence or directly, by peptide bonds. When linking the target protein to the anti-hTfR antibody light chain on the N-terminal side (or the C-terminal side) thereof, the C-terminus (or the N-terminus), respectively, of the target protein is linked to the N-terminus (or the C-terminus) of the λ chain and κ chain of anti-hTfR antibody, via a linker sequence or directly, by peptide bonds.

However, in the case where the anti-hTfR antibody consists of the Fab region, or of the Fab region and the whole or part of the hinge region (Fab, $F(ab')_2$, and $F(ab')$), the target protein may be linked at the C-terminus (or the N-terminus) thereof and via a linker sequence or directly, to the N-terminus (or the C-terminus), respectively, of the heavy chain or light chain that constitutes the Fab, $F(ab')_2$ and $F(ab')$, by peptide bonds.

[0082] In a fusion protein of the anti-hTfR antibody and a target protein, where the anti-hTfR antibody is a single-chain antibody, the amino acid sequence including the whole or part of the immunoglobulin light chain variable region and the amino acid sequence including the

whole or part of the immunoglobulin heavy chain variable region are linked, generally via a linker sequence. Insofar as the affinity of the anti-hTfR antibody to hTfR is retained, the amino acid sequence derived from the light chain may be linked, on the C-terminal side thereof, to a linker sequence which in turn being linked, on the C-terminal side thereof, to the amino acid sequence derived from the heavy chain or, conversely, the amino acid sequence derived from the heavy chain may be linked, on the C-terminal side thereof, to a linker sequence which in turn being linked, on the C-terminal side thereof, to the amino acid sequence derived from the light chain. The same can be established when the single-chain antibody is scFv.

[0083] In a single-chain antibody, the linker sequence placed between the light chain and the heavy chain of the immunoglobulin is preferably a peptide chain consisting of preferably 2 to 50, more preferably 8 to 50, still more preferably 10 to 30, even more preferably 12 to 18, or 15 to 25, for example 15 or 25 amino acid residues. Such a linker sequence is preferably, but not limited to, that made of glycine only or of glycine and serine, for example, amino acid sequence Gly-Ser, amino acid sequence Gly-Gly-Ser, amino acid sequence Gly-Gly-Gly, amino acid sequence Gly-Gly-Gly-Gly-Ser (SEQ ID NO: 3), amino acid sequence Gly-Gly-Gly-Gly-Gly-Ser (SEQ ID NO: 4), or a sequence in which these amino acid sequences are repeated for 2 to 10 or 2 to 5 times , as far as the anti-hTfR antibody to which both chains are linked retains affinity for hTfR and the target protein bound to the antibody exhibits its physiological activity under physiological conditions. As a preferable embodiment of the linker sequence, there is a sequence consisting of 15

amino acids in which the amino acid sequence Gly-Gly-Gly-Gly-Ser (SEQ ID NO: 3) is repeated for three times.

[0084] Where the anti-hTfR antibody is a single-chain antibody, such a fusion protein can be produced by, for example, transforming host cells such as mammalian cells with an expression vector having an incorporated DNA fragment containing a nucleotide sequence encoding the fusion protein, and then culturing the host cells.

[0085] Besides, in the present invention, when a peptide chain includes a plurality of linker sequences, each of those linker sequences is designated, from the N-terminal side, the first linker sequence, the second linker sequence, and so on, for convenience.

[0086] The target protein to be fused with the anti-hTfR antibody is not particularly limited insofar as the protein is to function in muscle. Preferred target protein includes, for example, a lysosomal enzyme encoded by a gene responsible for lysosomal diseases that cause muscle dysfunction. Such a lysosomal enzyme includes human acid α -glucosidase (hGAA) and human α -galactosidase A, but is not limited thereto, also includes α -L-iduronidase (IDUA), iduronate-2-sulfatase (IDS), glucocerebrosidase (GBA), β -galactosidase, GM2 activator protein, β -hexosaminidase A, β -hexosaminidase B, N-acetylglucosamine-1-phospholansferase, α -mannosidase (LAMAN), β -mannosidase, galactosylceramidase (GALC), saposin C, arylsulfatase A (ARSA), α -L-fucosidase (FUCA1), aspartylglucosaminidase, α -N-acetylgalactosaminidase, acid sphingomyelinase (ASM), β -glucuronidase (GUSB), heparan N-sulfatase (SGSH), α -N-acetylglucosaminidase (NAGLU),

acetyl-CoA: α -glucosaminide N-acetyltransferase, N-acetylglucosamine-6-sulfate sulfatase, acid ceramidase (AC), amylo-1,6-glucosidase, sialidase, aspartyl glucosaminidase, palmitoyl protein thioesterase-1 (PPT-1), tripeptidyl peptidase-1 (TPP-1), hyaluronidase-1, CLN1, CLN2, and the like.

5 [0087] Hereinafter, a fusion protein of an anti-hTfR antibody and a lysosomal enzyme will be described in detail by taking acid α -glucosidase and α -galactosidase A as examples.

[0088] When administered to a Pompe disease patient, the fusion protein of the anti-hTfR antibody and human acid α -glucosidase (hGAA) is incorporated into the patient's muscle cells and transported to lysosomes. Thus, it degrades glycogen accumulated in lysosomes of muscle cells of the patient with Pompe disease. In the case of Pompe disease, the target is the muscles consisting mainly of skeletal muscle cells and the muscles consisting mainly of heart muscle cells. In Pompe disease, the functional impairment of the heart and skeletal muscles is the main symptom, but by administering this fusion protein, the glycogen accumulated in the skeletal muscle cells and heart muscle cells is decomposed, which results in the improvement of symptoms such as 10 forcibly expand, muscle weakness based on skeletal muscle invasion, and weakness of muscle tension. Thus, the fusion protein of the anti-hTfR antibody and human acid α -glucosidase can be used as a therapeutic agent for skeletal muscular dysfunction or/and cardiac 15 disorders in Pompe disease.

20 [0089] Suitable examples of fusion proteins of anti-hTfR antibody and human acid α -glucosidase include a fusion protein of human acid

5 α -glucosidase and anti-hTfR antibody, wherein the light chain of the humanized anti-hTfR antibody has the amino acid sequence set forth as SEQ ID NO: 24, and wherein the heavy chain of the humanized anti-hTfR antibody is linked to human acid α -glucosidase on its C-terminal side via a linker sequence Gly-Ser, and the whole has the amino acid set forth as SEQ ID NO: 27.

10 [0090] Another example is a fusion protein of a human acid α -glucosidase and an anti-hTfR antibody, the fusion protein comprising a light chain of an anti-hTfR antibody having the amino acid sequence set forth as SEQ ID NO: 24, and a heavy chain of an anti-hTfR antibody having the amino acid sequence set forth as SEQ ID NO: 25 and linked on its C-terminal side to the human acid α -glucosidase set forth as SEQ ID NO: 1 via a linker sequence (Gly Ser).

15 [0091] When administered to a Fabry disease patient, the fusion protein of the anti-hTfR antibody and human α -galactosidase A (α -GalA) is incorporated into the patient's muscle cells and transported to lysosomes. Thus, it degrades trihexosylceramide accumulated in lysosomes of muscle cells of the patient with Fabry disease. In the case of Fabry disease, the target is the muscles consisting mainly of heart muscle cells. In Fabry disease, cardiovascular abnormalities are found, but by administering this fusion protein, the trihexosylceramide accumulated in heart muscle cells is decomposed, which can improve various symptoms of cardiovascular abnormalities such as ventricular expansion. This fusion protein is particularly effective for cardiac Fabry disease, an atypical type of Fabry disease in which the heart is primarily damaged. Thus, the fusion protein of the anti-hTfR antibody and human

α -galactosidase A can be used as a therapeutic agent for cardiac disorders in Fabry disease, particularly cardiac Fabry disease.

[0092] Suitable examples of fusion proteins of anti-hTfR antibody and human α -galactosidase A include a fusion protein of human α -galactosidase A and anti-hTfR antibody, wherein the light chain of the humanized anti-hTfR antibody has the amino acid sequence set forth as SEQ ID NO: 24, and wherein the heavy chain of the humanized anti-hTfR antibody is linked to human α -galactosidase A on its C-terminal side via linker sequence Gly-Ser, and the whole has the amino acid sequence set forth as SEQ ID NO: 29.

[0093] Another example is a fusion protein of a human acid α -glucosidase and an anti-hTfR antibody, the fusion protein comprising a light chain of an anti-hTfR antibody having the amino acid sequence set forth as SEQ ID NO: 24, and a heavy chain of an anti-hTfR antibody having the amino acid sequence set forth as SEQ ID NO: 25 and linked on its C-terminal side to the human α -galactosidase A set forth as SEQ ID NO: 2 via a linker sequence (Gly Ser).

[0094] The conjugate of an anti-hTfR antibody and a pharmaceutical agent can be used as a pharmaceutical composition to be administered into the blood to exert a therapeutic effect in muscle. Such a drug is generally administered to a patient by intravenous injection such as intravenous drip injection, subcutaneous injection, intramuscular injection, or the like, but the route of administration is not particularly limited.

[0095] The conjugate of the anti-hTfR antibody and the agent of the present invention can be provided to a medical institution as a

pharmaceutical composition in the form of a lyophilized product, an aqueous solution, or the like. In the case of the aqueous solution, the pharmaceutical composition may be provided as a formulation sealed in a vial or syringe, pre-dissolved in a solution containing a stabilizer, 5 buffer, isotonic agent. The formulation sealed in a syringe is commonly referred to as a prefilled syringe formulation. The prefilled syringe formulation can simplify the administration of the drug by the patient himself.

[0096] Where an aqueous preparation is provided, the concentration of 10 the conjugate in the aqueous preparation is, e.g., 1-4 mg/mL, though it is to be adjusted as desired in accordance with the dosage. Where there is no particular limitation as to stabilizers to be contained in the aqueous preparation insofar as they are pharmaceutically available, nonionic surfactants may preferably be used. Examples of such 15 nonionic surfactants include polysorbate and poloxamer, either of which may be used alone or in combination. Among polysorbates, polysorbate 20 and polysorbate 80 are preferably used. As poloxamer, poloxamer 188 (polyoxyethylene (160) polyoxypropylene (30) glycol) is particularly preferred. Further, the concentration of nonionic 20 surfactant contained in the aqueous preparation is preferably 0.01-1 mg/mL, more preferably, 0.01-0.5 mg/mL, and still more preferably 0.1-0.5 mg/mL. As stabilizers, amino acids such as histidine, arginine, methionine, and glycine may also be used. Where employed as a stabilizer, the concentration of an amino acid in the aqueous preparation 25 is preferably 0.1-40 mg/mL, more preferably 0.2-5 mg/mL, and still more preferably 0.5-4 mg/mL. While there is no particular limitation

as to a buffer to be contained in the aqueous preparation insofar as it is pharmaceutically available, phosphate buffer is preferred, and more preferred is sodium phosphate buffer. Where used as a buffer, the concentration of sodium phosphate is preferably 0.01-0.04 M. The pH 5 of the aqueous preparation adjusted with a buffer is preferably 5.5-7.2. While there is no particular limitation as to an isotonizer to be contained in the aqueous preparation insofar as it is pharmaceutically available, sodium chloride or mannitol may be preferably used alone or in combination as an isotonizer.

10 **Examples**

[0097] Though the present invention is described in further detail below with reference to examples, it is not intended that the present invention be limited to those examples.

[0098] [Example 1] Construction of hTfR expression vector

15 Employing human spleen Quick Clone cDNA (Clontech Inc.) as a template and using primer hTfR5' (SEQ ID NO:7) and primer hTfR3' (SEQ ID NO:8), PCR was performed to amplify the gene fragment encoding human transferrin receptor (hTfR). The amplified fragment encoding hTfR was digested with MluI and NotI, and then inserted 20 between MluI and NotI sites of vector pCI-neo (Promega Inc.). The vector thus prepared was designated pCI-neo(hTfR). This vector then was digested with MluI and NotI to cut out the gene fragment encoding hTfR, and this fragment was inserted between MluI and NotI sites of pE-mIRES-GS-puro, an expression vector disclosed in an international publication WO 2012/063799 to construct an hTfR expression vector, 25 pE-mIRES-GS-puro(hTfR).

[0099] [Example 2] Preparation of recombinant hTfR

Into CHO-K1 cells was introduced pE-mIRES-GS-puro(hTfR) by electroporation, and the cells then were subjected to selection culture in a CD OptiCHO™ medium (Invitrogen Inc.) containing methionine sulfoximine (MSX) and puromycin to prepare recombinant hTfR expressing cells. The recombinant hTfR expressing cells were cultured, and recombinant hTfR was prepared.

[0100] [Example 3] Immunization of mouse with recombinant hTfR

Mice were immunized with recombinant hTfR prepared in Example 2 as antigen. Immunization was carried out by intravenously or intraperitoneally injecting the mice with the antigen.

[0101] [Example 4] Preparation of hybridomas

About one week after the last injection, the spleens of the mice were excised and homogenized to isolate spleen cells. The spleen cells thus obtained were fused with cells of mouse myeloma cell line (P3.X63.Ag8.653) by the polyethylene glycol method. After cell fusion, the cells were suspended in a RPMI 1640 medium containing (1X) HAT supplement (Life Technologies Inc.) and 10% Ultra low IgG fetal bovine serum (Life Technologies Inc.), and the cell suspension was dispensed to 20 of 96-well plates, 200 µL/well. After the cells were cultured for 10 days in a carbon dioxide gas incubator (37°C, 5% CO₂), each well was examined under a microscope, and the wells that contain a single colony were selected. When the cells in each well reached near confluence, the culture supernatant was collected as a culture supernatant of hybridoma, and subjected to the following screening process.

[0102] [Example 5] Screening of high affinity antibody producing cell line

The recombinant hTfR solution (Sino Biologics Inc.) was diluted with 50 mM sodium phosphate buffer (pH 9.5-9.6) to 5 μ g/mL to prepare a solid phase solution. After 50 μ L of the solid phase solution was added to each well of a Nunc MaxiSorpTM flat-bottom 96-well plate (substrate: polystyrene, mfd. by Nunc Inc.), the plate was left to stand for one hour at room temperature to let the recombinant hTfR adhere to the plate and become immobilized. The solid phase solution was discarded, each well was washed three times with 250 μ L of washing solution (PBS containing PBS-T: 0.05% Tween20), 200 μ L of a blocking solution (PBS containing 1% BSA) then was added to each well, and the plate was left to stand for one hour at room temperature.

[0103] The blocking solution was discarded, and each well was washed three times with 250 μ L of PBS-T. To each well was added 50 μ L of the hybridoma culture supernatant, and the plate was left to stand for one hour at room temperature to let the mouse anti-hTfR antibody contained in the culture supernatant bind to the recombinant hTfR. At the same time, to some wells was added 50 μ L of culture supernatant of a hybridoma that did not produce mouse anti-hTfR antibody, as a control. In addition, 50 μ L of the medium for hybridoma culture was added to the wells, as mock wells, beside those wells to which the culture supernatant was added. Measurement was conducted in an n=2 fashion. Then, the solution was discarded, and each well was washed three times with 250 μ L of PBS-T.

[0104] To each of the above wells was added 100 μ L of HRP-labeled goat anti-mouse immunoglobulin antibody solution (Promega Inc.), and the plate was left to stand for one minute at room temperature. The solution then was discarded, and each well was washed three times with 5 250 μ L of PBS-T. To each well was added 50 μ L of a chromogenic substrate solution, TMB Stabilized Substrate for Horseradish Peroxidase (Promega Inc.), and the wells were left to stand for 10 to 20 minutes at room temperature. Then, following addition of 100 μ L of a stop solution (2N sulfuric acid), the absorbance of each well was measured 10 on a plate reader at 450 nm. Of the two wells for each of the culture supernatant and control, the mean values were taken, respectively, and from each of the mean values, the respective mean value for the two mock wells placed corresponding to each of the culture supernatant and the control, was subtracted, giving the measurement.

15 [0105] Fourteen types of hybridoma cells corresponding to culture supernatants added to the wells which exhibited the higher measurements were selected as the cell lines (high affinity antibody producing cell line) that produce antibodies exhibiting high affinities to hTfR (high affinity anti-hTfR antibody). These fourteen types of cell 20 lines were designated as Clone 1 line to Clone 14 line. Anti-hTfR antibodies produced by clonal cell lines 1 to 14 were designated as anti-hTfR antibody No. 1 to anti-hTfR antibody No. 14, respectively.

[0106] [Example 6] Analysis of the variable-region amino acid sequence of the high affinity anti-hTfR antibodies

25 Further clone 3 line was selected from clone 1 line to clone 14 line selected in Example 5. A cDNA of clone 3 line was prepared, and

the gene encoding the light chain and heavy chain of an antibody was amplified using this cDNA as a template. The nucleic acid sequence of the amplified gene was translated to determine the amino acid sequence of the variable region of the light chain and heavy chain of the 5 anti-hTfR antibody No. 3 produced by this clone line.

[0107] The anti-hTfR antibody No.3 was found to include the amino acid sequence set forth as SEQ ID NO:9 as the light chain variable region, and the amino acid sequence set forth as SEQ ID NO:10 as the heavy chain variable region. The light chain variable region was found 10 to include the amino acid sequence set forth as SEQ ID NO:11 or 12 as CDR1; SEQ ID NO:13 or 14 as CDR2, and SEQ ID NO:15 as CDR3; and the heavy chain variable region to include the amino acid sequence set forth as SEQ ID NO:16 or 17 as CDR1, SEQ ID NO:18 or 19 as CDR2, and SEQ ID NO:20 or 21 as CDR3. However, it was also 15 considered that CDRs are not limited to those which consist of these amino acid sequences, but they can also either be regions of amino acid sequences that include any of the above sequences, or amino acid sequences consisting of not less than three consecutive amino acids containing part of the above sequences.

20 [0108] [Example 7] Measurement of the affinity of anti-hTfR antibody to human and monkey TfRs

The affinity of the anti-hTfR antibody to human and monkey TfRs were measured on Octet RED96 (ForteBio Inc., a division of Pall Corporation), a system for analysis of interactions between 25 biomolecules utilizing bio-layer interferometry (BLI). The basic principles of bio-layer interferometry are briefly explained below.

When a layer of a biomolecule immobilized on the surface of a sensor tip is irradiated with light of a certain wavelength, the light is reflected from two of the surfaces, the one of the biomolecule and the other of inner, reference layer, producing interfering light waves. A molecule in the sample being measured binds to the biomolecule on the surface of the sensor tip and thus increases the thickness of the layers on the sensor tip, which results in a shift between the interfering waves. By measuring the variations of this shift between the interfering waves, determination of the number of the molecules bound to the layer of the biomolecules immobilized to the sensor tip surface and kinetic analysis of it can be performed in real time. The measurement was performed according generally to the operating manual attached to Octet RED96. As a human TfR, a recombinant human TfR (r human TfR: Sino Biological Inc.) was used, which had the amino acid sequence of the hTfR extracellular region, i.e., the cysteine residue at position 89 from the N-terminal side to the phenylalanine at the C-terminus, of the amino acid sequence set forth as SEQ ID NO:1, with a histidine tag attached to the N-terminus. As a monkey TfR, a recombinant monkey TfR (r monkey TfR: Sino Biological Inc.) was used, which had the amino acid sequence of TfR extracellular region of *Macaca fascicularis*, i.e., the cysteine residue at position 89 from the N-terminal side to the phenylalanine at the C-terminus, of the amino acid sequence set forth as SEQ ID NO:2, with a histidine tag attached to the N-terminus.

[0109] Clone 3 line selected in Example 6 was diluted with a RPMI 1640 medium containing (1X) HAT Supplement (Life Technologies Inc.) and 10% Ultra low IgG fetal bovine serum (Life Technologies

Inc.) so as to adjust the cell density to approximately 2×10^5 cells/mL. To a 1 L conical flask were added 200 mL of each cell suspension, and the culture was performed for 6 to 7 days in a wet environment at 37°C, 5% CO₂ and 95% air, with stirring at a rate of about 70 rpm. The 5 culture supernatant was centrifuged, and then filtered through a 0.22 µm filter (Millipore Inc.) to collect a culture supernatant. The culture supernatant thus collected was loaded onto a Protein G column (column volume: 1 mL, GE Healthcare Inc.) that had been equilibrated in advance with three column volumes of 20 mM Tris buffer (pH 8.0) 10 containing 150 mM NaCl. After the column was washed with 5 column volumes of the same buffer, adsorbed antibody was eluted with 4 column volumes of 50 mM glycine buffer (pH 2.8) containing 150 mM NaCl, and eluted fractions were collected. The fractions eluted were adjusted to pH 7.0 by addition of 1 M Tris buffer (pH 8.0). These 15 were used as purified products of anti-hTfR antibody No. 3 in the experiments described below.

[0110] Purified anti-hTfR antibody No. 3 was diluted in two steps with HBS-P + (10mM HEPES containing 150 mM NaCl, 50 µM EDTA and 0.05% Surfactant P20), respectively, to prepare antibody solutions of 7 20 different concentrations, 0.78125 to 50 nM (0.117 to 7.5 µg/mL). These antibody solutions were used as sample solutions. The r human and r monkey TfRs were respectively diluted with HBS-P+ to prepare 25 µg/mL solutions, which were used as r human TfR-ECD (Histag) solution and r monkey TfR-ECD (Histag) solution, respectively.

25 [0111] Each of the sample solutions prepared above by 2-fold dilution steps was added, 200 µL/well, to a 96-well plate, black (Greiner

Bio-One Inc.). Each of the r human TfR-ECD (Histag) solution and the r monkey TfR-ECD (Histag) solutions prepared above was added, 200 μ L/well, to predetermined wells. To respective wells for baseline, dissociation and washing were added HBS-P+, 200 μ L/well. To wells for regeneration were added 10 mM Glycine-HCl (pH 1.7), 200 μ L/well. To wells for activation was added 0.5 mM NiCl₂ solution, 200 μ L/well. The plate and biosensor (Biosensor/Ni-NTA: ForteBio Inc., a division of Pall Corporation) were set in the prescribed positions of Octet RED96.

[0112] Octet RED96 was run under the conditions shown in Table 1 below to collect data, on which then, using the analyzing software attached to Octet RED96, and fitting the binding reaction curve to 1:1 binding model or 2:1 binding model, the association rate constant (k_{on}) and dissociation rate constant (k_{off}) of anti-hTfR antibody to r human TfR and r monkey TfR were measured and the dissociation constant (K_D) was calculated. The measurement was performed at 25 to 30°C.

[0113] [Table 1]

Table 1 Operating Conditions of OctetRED96

	Step	Sensor contact time (sec)	Rate (rpm)	Threshold
1	Base line 1	60	1000	-
2	Loading	600	1000	1.5 – 2.0
3	Base line 2	60	1000	-
4	Association	180	1000	-
5	Dissociation	540	1000	-
6	Regeneration	5	1000	-
7	Washing	5	1000	-
The steps 6 to 7 above are repeated for 6 to 7 times.				
8	Activation	60	1000	-
The steps 1 to 8 are repeated until all samples have been measured.				

[0114] The dissociation constant (K_D) with human TfR of anti-hTfR No. 3 was not more than 1×10^{-12} M, and the dissociation constant (K_D) with monkey TfR was not more than 1×10^{-12} M. These results indicate that anti-hTfR antibody No. 3 is an antibody with higher affinity not only for human TfR but also for monkey TfR.

[0115] [Example 8] Preparation of humanized anti-hTfR antibodies

Humanization was tried of the amino acid sequence included in the light chain and the heavy chain variable regions of anti-hTfR antibody No. 3. Thus the variable region of the humanized light chain having the amino acid sequence set forth as SEQ ID NO: 22 and the variable region of the humanized heavy chain having the amino acid sequence set forth as SEQ ID NO: 23 were obtained. Further, for the light chain, a light chain having the amino acid sequence set forth as SEQ ID NO: 24 and having the amino acid sequence set forth as SEQ ID NO: 22 in the variable region was obtained. As for the heavy chain, a

heavy chain having the amino acid sequence set forth as SEQ ID NO: 25 and having the amino acid sequence set forth as SEQ ID NO: 23 in the variable region was obtained. The heavy chain of the amino acid sequence set forth as SEQ ID NO: 25 is of IgG4 type. The dissociation factor (K_D) of the humanized anti-hTfR antibody with human TfR thus obtained was measured by the method described in Example 7. The results show that the dissociation constant (K_D) of humanized anti-hTfR antibody with human TfR was not more than 1×10^{-12} M, and the dissociation constant (K_D) with monkey TfR was about 1×10^{-9} M.

5 These results indicate that the obtained humanized antibody of anti-hTfR antibody No. 3 has high affinity not only for human TfR but also for monkey TfR.

10

[0116] [Example 9] Construction of a gene encoding the fusion protein of anti-TfR antibody and hGAA

15 A DNA fragment encoding the light chain of the amino acid sequence set forth as SEQ ID NO: 24 (SEQ ID NO: 26) was synthesized. The fragment has, on the 5' side, in order from the 5' end, a MluI sequence and a sequence encoding a leader peptide which functions as a secretory signal, and a NotI sequence on the 3' side.

20 [0117] In addition, a DNA fragment (SEQ ID NO: 28) encoding a protein of the amino acid sequence set forth as SEQ ID NO: 27 as a whole was synthesized in that protein a human acid α -glucosidase of the amino acid sequence set forth as SEQ ID NO: 1 was linked on the C-terminal side of the heavy chain of the amino acid sequence set forth as SEQ ID NO: 25 via a linker sequence consisting of Gly-Ser. The DNA fragment has, on the 5' side, in order from the 5' end, a MluI

25

sequence and a sequence encoding a leader peptide which functions as a secretory signal, and a NotI sequence on the 3' side.

[0118] [Example 10] Construction of an expression vector for fusion protein of anti-TfR antibody and hGAA

5 A pEF/myc/nuc vector (Invitrogen Inc.) was digested with KpnI and NcoI, the region containing the EF-1 α promoter and its first intron was excised and blunt-ended with T4 DNA polymerase. A pCI-neo vector (Invitrogen Inc.) was digested with BglII and EcoRI to excise the region containing CMV enhancer/promoter and intron, followed by 10 blunt-end treatment with T4 DNA polymerase. Into this, the region containing the EF-1 α promoter and its first intron described above was inserted to construct a pE-neo vector. A pE-neo vector was digested with SfiI and BstXI, and approximately 1 kbp region containing neomycin 15 resistance gene was excised. Using PcdNA3.1/Hygro(+) (Invitrogen Inc.) as a template, Hygromycin resistance gene was amplified by PCR-reaction using primer Hyg-Sfi5' (SEQ. NO. 30) and primer Hyg-BstX3' (SEQ. NO. 31). The amplified hygromycin resistance gene was digested with SfiI and BstXI, and the neomycin resistance gene was inserted into the excised pE-neo vector to construct a pE-hygr vector.

20 [0119] A pE-hygr vector and a pE-neo vector were digested with MluI and NotI, respectively. The DNA fragment encoding the light chain of the anti-hTfR antibody synthesized in Example 9 (SEQ ID NO: 26) and the DNA fragment encoding the protein in which the heavy chain of the anti-hTfR antibody and human acid α -glucosidase were linked (SEQ ID NO: 28) were digested with MluI and NotI, and inserted between 25 MluI-NotI of pE-hygr vector and pE-neo vector, respectively. The

obtained vectors were used as a pE-hygr (LC), a vector for expression of light chain of a humanized anti-hTfR antibody, and a pE-neo(HC-hGAA, a vector for expression of protein in which a heavy chain of an anti-hTfR antibody and human acid α -glucosidase were linked, respectively.

5 [0120] [Example 11] Preparation of cells for expressing hGAA-humanized anti-hTfR antibody fusion protein

CHO cells (CHO-K1: obtained from American Type Culture Collection) we transformed with pE-hygr (LC) and pE-neo(HC-hGAA) constructed in Example 10 using GenePulser(Bio-Rad, Inc.) according to the method described below. The transformation of the cell was generally carried out in the following manner. 5X10⁶ of CHO-K1 cells were seeded in a 3.5 cm culture dish with CD OptiCHOTM medium (Life Technologies Inc.) added, and were grown overnight under the conditions of 37°C and 5% CO₂. The medium was exchanged for Opti-MEMTM I medium (Life Technologies Inc.) and the cells were suspended at the density of 5X10⁶ cells/mL. 100 μ L of the cell suspension was collected, and 5 μ L each of the pE-hygr(LC1) plasmid DNA solution and the pE-neo(HC1) plasmid DNA solution, both diluted to 100 μ g/mL in Opti-MEM mediumTM I medium, were added to the cell suspension. Electroporation was performed using GenePulser (Bio-Rad Inc.) and plasmids were introduced into the cells. The cells then were cultured overnight under the condition of 37°C, 5% CO₂, and subjected to selection culture in CD OptiCHOTM medium supplemented with 0.5 mg/mL of hygromycin and 0.8 mg/mL of G418.

20 [0121] Then, the cells selected above through the selection culture were

seeded on 96-well plates so that not more than one cell was seeded per well by limiting dilution. The cells then were cultured for about 10 days so that monoclonal colonies were formed. Respective culture supernatants of the wells in which monoclonal colony was formed were 5 collected, the amount of the humanized antibody contained in culture supernatants was determined by ELISA, and hGAA-humanized anti-hTfR antibody-fusion protein high-expressing cell lines were selected.

[0122] The ELISA above was conducted as follows in general. To 10 each well of 96-well microtiter plates (Nunc Inc.) were added 100 μ L of a goat anti-human IgG polyclonal antibody solution diluted with 0.05 M sodium bicarbonate buffer (pH 9.6) to 4 μ g/mL, and the plate was left to stand for at least one hour at room temperature so as to allow the antibody to be adsorbed by the plates. Then, after the well were 15 washed three times with PBS-T, 200 μ L of Starting Block (PBS) Blocking Buffer (Thermo Fisher Scientific Inc.) was added to each well, and the plates were left to stand for 30 minutes at room temperature. After each well was washed with PBS-T three times, the culture supernatant or the human IgG standard product which had been diluted 20 with a PBS-BT (PBS supplemented with 0.5% BSA and 0.05% Tween20) to appropriate concentrations, was added to each well, in the amount of 100 μ L, and the plates were left to stand for at least one hour at room temperature. After the plates were washed three times with PBS-T, 100 μ L of HRP-labeled anti-human IgG polyclonal antibody 25 solution which had been diluted with PBS-BT, was added to each well, and the plates were left to stand for at least one hour at room

temperature. After the wells were washed three times with PBS-T, 0.4 mg/mL o-phenylenediamine in citrate-phosphate buffer (pH 5.0) was added to each well, in the amount of 100 μ L, and the wells were left to stand for 8 to 20 minutes at room temperature. Then, 1 mol/L sulfuric acid was added to each well, in the amount of 100 μ L to terminate the reaction, and the absorbance for each well was measured at 490 nm using a 96-well plate reader. The cells corresponding to the wells which exhibited the higher measurements were regarded as a hGAA-humanized anti-hTfR antibody-fusion protein high-expressing cell line. The hGAA-humanized anti-hTfR antibody-fusion protein produced by this cell line was designated as hGAA-anti-hTfR antibody.

10 [0123] [Example 12] Production of the hGAA-Anti-hTfR Antibody

The hGAA-anti-hTfR antibody was produced in the following manner. The high-expressing cell line of the hGAA-anti-hTfR antibody obtained in Example 11 was diluted in CD OptiCHOTM medium to a cell concentration of about 2×10^5 cells/mL, 200 mL of cell suspension was added to 1 L Erlenmeyer flask, and incubated at 37°C in a wet environment consisting of 5% CO₂ and 95% air at a stirring rate of about 70 rpm for 6-7 days. The culture supernatant was collected by centrifugation and filtered through a 0.22 μ m filter (Millipore Inc.) to obtain a culture supernatant. To the harvested culture supernatant was added 5 column volumes of 20 mM Tris buffer (pH 8.0) containing 150 mM NaCl and it was loaded onto a Protein A column (column volume: 1 mL, Bio-Rad Inc.) that had been pre-equilibrated with 3 column volumes of 20 mM Tris buffer (pH 8.0) containing 150 mM NaCl. The column was then washed with 5 column volumes of the same buffer and

the adsorbed hGAA-anti-hTfR antibody was eluted with 4 column volumes of 50 mM glycine buffer (pH 2.8) containing 150 mM NaCl. The pH of this eluate containing the hGAA-anti-hTfR antibody was adjusted to pH 7.0 by adding 1 M Tris buffer (pH 8.0), and then buffer-exchanged to PBS using an Amicon Ultra 30 kDa membrane (Millipore Co.). Thus obtained was used as a purified product of the hGAA-anti-hTfR antibody.

5 [0124] [Example 13] Measurement of incorporation of hGAA into cells by using human skeletal muscle

10 Human skeletal myocytes (PromoCell Inc.) are diluted to 8.0X10⁴ cells/mL by Skeletal Muscle Cell Growth Medium (Ready-to-use) (PromoCell Inc.) and seeded into the wells of a 96 well plate coated with CellnestTM (Fujifilm Inc.) in 250 µL/well. After culturing at 37°C in 5% CO₂ for 3 days, the medium is replaced with 15 medium for inducing skeletal myocyte differentiation, Skeletal Muscle Cell Differentiation Medium (Ready-to-use) (PromoCell Inc.). Then, every 2 days, the medium is changed with the medium for induction of differentiation, and differentiation is induced for 6 days. After removing the medium, 100 µL of each of serially diluted solutions of the 20 hGAA-anti-hTfR antibody and hGAA (0.005-20.0 µg/mL), diluted with the medium for inducing differentiation, is added to the well as a test substance, and the cells is further incubated at 37°C and 5% CO₂ for an additional 20 hours.

25 [0125] After incubation, the medium is removed and each well is washed three times with 300 µL of PBS. 100 µL of M-PERTM (with 0.5 % Protease Inhibitor Cocktail, Thermo Fisher Scientific Inc.) is

added to each well, and the plate is shaken by a plate shaker at room temperature for 25 minutes to prepare a cellular extract. 20 μ L of the extract from each well is collected and the protein contained in the extract is determined by using BCA protein assay kit (Pierce Inc.). In 5 addition, 80 μ L of PBS-BT is added to the wells after collecting 20 μ L of the extract, and the plate is shaken for 10 minutes at room temperature on a plate shaker to prepare a cellular extract to be subjected to ELISA measurement.

10 [0126] A cell extract is prepared in the same manner from cells in wells to which the test substance is not added, and this extract is used as a pool extract. The hGAA-anti-hTfR antibody and commercial available hGAA are serially diluted with pooled extracts to prepare calibration curve samples. The test substance can be quantitated by the following ELISA method.

15 [0127] Rabbit anti-hGAA polyclonal antibody obtained by immunizing a rabbit with hGAA are diluted to 5 μ g/mL with 20 mmol/L sodium carbonate buffer (pH 9.0), added to each well of a 96 well plate (MaxisorpTM) in 100 μ L /well, and shaken for 1 hour at room temperature on a plate shaker. The antibody solution is removed and 20 300 μ L of blocking buffer (PBS with 1% BSA) is added to each well and left to stand for 1 hour at room temperature. The wells are then washed three times with 300 μ L of PBS-T. And 100 μ L of each of the standard curve sample and cell extract is added to the well, and the plate is shaken for 1 hour at room temperature on a plate shaker. Each well is 25 washed three times with 300 μ L of wash solution, 100 μ L of Biotin labeled rabbit anti-hGAA antibody (prepared in-house) in PBS-BT is

added to each well, and the plate is shaken on a plate shaker at room temperature for 1 hour. After washing each well three times with 300 μ L of wash solution, 100 μ L of NeutrAvidin-HRP conjugate (Pierce Inc.) diluted with 0.5% BSA/PBS (with 0.05% Tween20) is added to each well, the plate is shaken for 15 minutes at room temperature on a plate shaker, and then each well is washed three times with 300 μ L of wash solution. The mixed solution of TMB Peroxidase Substrate Solution B of TMB Microwell Peroxidase Substrate (2-Component System, KPL Inc.) and TMB Peroxidase Substrate in equal amounts is added to each well in 100 μ L/well and a color reaction is developed at room temperature. After adequate color development is obtained, 100 μ L of 1 mol/L phosphate is added to each well and the reaction is stopped. The absorbance at 450 nanometers is measured with a microplate reader, a calibration curve is prepared by using SoftMax Pro (Molecular Device Inc.), and the concentrations of each test substance are calculated by interpolating the measured values of each test substance to the calibration curve. The concentration of the test substance in each cell extract is divided by the protein concentration to obtain the amount of the test substance taken into the cell (ng/mg protein).

[0128] [Example 14] Preparation of CD71-KI/GAA-KO mice

CD71-KI/GAA-KO mice knocking out the acid α -glucosidase gene (GAA gene) and knocking in the human transferrin receptor gene (CD71 gene) were generated by common genetic engineering techniques. The production method was generally as follows. A targeting vector (GAA knockout vector) capable of knocking out the mouse GAA gene was constructed by inserting a stop codon and a

neomycin resistance gene into BamHI site present in exon 6 of the mouse GAA gene. The targeting vector comprises the nucleic acid sequence set forth as SEQ ID NO: 32. After linearization of the targeting vector, the vector was introduced into the ES cells of C57BL/6 lineage by electroporation. After the introduction, selective culture using neomycin drug resistance as an index was carried out, and drug-resistant ES clones were obtained. The obtained drug-resistant ES clones were screened by genomic PCR and Southern blot, and clones in which specific homologous recombination was achieved (homologous recombination ES clones) were selected. Using homologous recombination ES clones and 8-cell stage embryos of ICR lineage, chimeric embryos were produced by aggregation method. Recipient mouse was implanted with chimeric embryos to deliver chimeric mice.

[0129] The chimeric mice were naturally mated to produce GAA-KO hetero mice, and GAA-KO homo mice were produced through repeated mating. CD71-KI hetero mice and GAA-KO homo mice were mated and the resulting mice were screened to produce CD71-KI/GAA-KO mice. CD71-KI hetero mice were prepared according to the method described in Sonoda H., et al. Molecular Therapy 26. 1366-74 (2018).

[0130] [Example 15] Measurement of hGAA incorporation into cells in mice

Purified product of hGAA-anti-hTfR antibody prepared in Example 12 and commercial available hGAA for medical use were each prepared as 4 mg/mL solutions using physiological saline. Using these solutions, CD71-KI/GAA-KO mice were administered hGAA-anti-hTfR antibody or hGAA via the tail veins, respectively.

Both hGAA-anti-hTfR antibody and hGAA were administered at a dose of 20 mg/dose for a total of 4 doses every other week. Two weeks after the last dose, the mice were exsanguinated to death, and the heart, diaphragm, soleus, tibialis anterior, and quadriceps muscles were 5 collected. The collected tissue was washed with saline. The concentrations of glycogens in these muscular tissues, mg/g wet tissue weights, were measured as described in Example 16. The group that received hGAA-anti-hTfR antibody was designated as the hGAA-anti-hTfR antibody-administered group and the group that received commercially available medical hGAA was designated as the 10 hGAA-administered group. CD71-KI/GAA-KO mice administered with an equal volume of saline were used as the KO-control group. In addition, wild-type mice that received an equal volume of saline were used as the wild-type control group. Each group consisted of 5 mice.

15 [0131] The results of the measurement of the amount of glycogen contained in each muscle tissue are shown in figures 1 to 5. Figure 1, 2, 3, 4, and 5 show the result of the measurement of the heart, the diaphragm, the soleus muscle, anterior tibial muscle, and the quadriceps muscle. When the amount of glycogen contained in each tissue in 20 hGAA-administered group was set as 100%, the amount of glycogen contained in each tissue in hGAA-anti-hTfR antibody-administered group decreased to approximately 7.5% in the heart, 6.5% in the diaphragm, 13.5% in the soleus, 13.5% in the anterior tibial muscle, and 6% in the quadriceps muscle. These results demonstrate that 25 hGAA-anti-hTfR antibody can efficiently degrade the glycogen accumulated in muscular tissue compared to commercially available

medical-grade hGAA. Pompe disease (glycogen storage disease type II) is a disease in which glycogen accumulates in large amounts in lysosomes in cells by genetically lacking most or all of the acid α -glucosidase activity, and the main symptom is dysfunction of cardiac and skeletal muscles. That is, hGAA-anti-hTfR antibody is promising as a therapeutic agent in enzymatic replacement therapy of Pompe disease patients because they can efficiently degrade glycogens accumulated in cardiac and skeletal muscles of Pompe disease patients compared to hGAA.

5 [0132] [Example 16] Method for determining the amount of glycogen contained in tissues

The tissue obtained in Example 15 and 15 pieces of 0.5 mm-diameter SUS beads were placed in a holder set in a bead crusher (BEADS CRUSHER μ T-12, Titec Inc.), and water for injection was 15 further added to the holder. The bead crusher was operated to crush the tissue. The obtained tissue crushed material was centrifuged at 15,000 rpm for 10 minutes at 4°C, and the supernatant was collected as a sample solution and stored frozen until the measurement.

[0133] Glycogen concentration was measured using Glycogen Colorimetric/Fluorometric Assay Kit (Biovision Inc.) in the following steps as a whole. The calibration curve sample (Glycogen Standard) was diluted stepwise with pure water, and the 50 μ L of diluted solutions were added to the wells of F96 Black plate (Nunc Inc.). In likewise, 50 μ L of the sample solution was added to each well. At this time, each of the 25 calibration curve sample diluted solutions and the sample solutions was added to two wells, respectively. 1 μ l of Hydrolysis Enzyme Mix was

added to two wells to which a calibration curve sample was added and to one well to which a sample was added each, mixed, and reacted at room temperature for 30 minutes to degrade glycogen into glucose. Then, 50 μ l of a Reaction Mix containing a fluorescent reagent was 5 added to all the wells and reacted for 30 minutes at room temperature in a light-proof manner. Fluorescence intensities were measured (Ex/Em = 535/587 nm) using a fluorescent plate reader (SPECTRA max GEMINI XPS, Molecular Devices Inc.) and the glucose concentration of the sample solution to which Hydrolysis Enzyme Mix was added and that 10 of the sample solution to which Hydrolysis Enzyme Mix was not added were calculated from a calibration curve prepared by Linear fit curve from the theoretical concentration (X) and the signal mean (Y) of the calibration curve samples. The glucose concentration measurement of the sample solution to which Hydrolysis Enzyme Mix was not added 15 was then subtracted from the glucose concentration measurement of the sample solution to which Hydrolysis Enzyme Mix was added, and this value was taken as the glycogen concentration of the sample solution. Glycogen concentrations (mg/g wet tissue weights) of the tissues were determined from the weights of the tissues used to prepare the sample 20 solutions and the determined glycogen concentrations.

Industrial Applicability

[0134] According to the present invention, a pharmaceutical agent having to function in muscle, which is not sufficiently taken up into muscle as it is, can be efficiently taken up into muscle by making it as a 25 conjugate coupled to an anti-transferrin receptor antibody. Therefore it can be applied to the development of a new therapeutic drug for muscle

disease.

Sequence Listing Free Text

[0135] SEQ ID NO:3: Amino acid sequence of an exemplified linker 1

SEQ ID NO:4: Amino acid sequence of an exemplified linker 2

5 SEQ ID NO:7: Primer hTfR5', synthetic sequence

SEQ ID NO:8: Primer hTfR3', synthetic sequence

SEQ ID NO:9: Amino acid sequence of the light-chain variable
region of mouse anti-hTfR antibody No. 3

10 SEQ ID NO:10: Amino acid sequence of the heavy-chain
variable region of mouse anti-hTfR antibody No. 3

SEQ ID NO:11: Amino acid sequence 1 of CDR1 in the light
chain of mouse anti-hTfR antibody No. 3

SEQ ID NO:12: Amino acid sequence 2 of CDR1 in the light
chain of mouse anti-hTfR antibody No. 3

15 SEQ ID NO:13: Amino acid sequence 1 of CDR2 in the light
chain of mouse anti-hTfR antibody No. 3

SEQ ID NO:14: Amino acid sequence 2 of CDR2 in the light
chain of mouse anti-hTfR antibody No. 3

20 SEQ ID NO:15: Amino acid sequence of CDR3 in the light
chain of mouse anti-hTfR antibody No. 3

SEQ ID NO:16: Amino acid sequence 1 of CDR1 in the heavy
chain of mouse anti-hTfR antibody No. 3

SEQ ID NO:17: Amino acid sequence 2 of CDR1 in the heavy
chain of mouse anti-hTfR antibody No. 3

25 SEQ ID NO:18: Amino acid sequence 1 of CDR2 in the heavy
chain of mouse anti-hTfR antibody No. 3

SEQ ID NO:19: Amino acid sequence 2 of CDR2 in the heavy chain of mouse anti-hTfR antibody No. 3

SEQ ID NO:20: Amino acid sequence 1 of CDR3 in the heavy chain of mouse anti-hTfR antibody No. 3

5 SEQ ID NO:21: Amino acid sequence 2 of CDR3 in the heavy chain of mouse anti-hTfR antibody No. 3

SEQ ID NO:22: Amino acid sequence of the light-chain variable region of humanized anti-hTfR antibody No. 3

10 SEQ ID NO:23: Amino acid sequence of the heavy-chain variable region of humanized anti-hTfR antibody No. 3

SEQ ID NO:24: Amino acid sequence of the light-chain of humanized anti-hTfR antibody No. 3

SEQ ID NO:25: Amino acid sequence of the heavy-chain of humanized anti-hTfR antibody No. 3

15 SEQ ID NO:26: Nucleic acid sequence encoding the amino acid sequence of the light-chain of humanized anti-hTfR antibody No. 3, synthetic sequence

SEQ ID NO:27: Amino acid sequence of the fusion protein of the heavy chain of humanized anti-hTfR antibody No. 3 and hGAA

20 SEQ ID NO:28: Nucleic acid sequence encoding the amino acid sequence of the fusion protein of the heavy chain of humanized anti-hTfR antibody No. 3 and hGAA, synthetic sequence

SEQ ID NO:29: Amino acid sequence of the fusion protein of the heavy chain of humanized anti-hTfR antibody No. 3 and h alfa-galA

25 SEQ ID NO:30: Primer Hyg-Sfi5', synthetic sequence

SEQ ID NO:31: Primer Hyg-BstX3', synthetic sequence

SEQ ID NO:32: Nucleic acid sequence of GAA knockout vector, synthetic sequence

CLAIMS

1. A conjugate of an anti-human transferrin receptor antibody and an agent, wherein the agent has a physiological activity to be exerted in muscle.

5 2. The conjugate according to claim 1, wherein the anti-human transferrin receptor antibody is a Fab antibody, a F(ab')₂ antibody, or a F(ab') antibody.

10 3. The conjugate according to claim 1, wherein the anti-human transferrin receptor antibody is a single-chain antibody selected from the group consisting of scFab, scF(ab'), scF(ab')₂, and scFv.

4. The conjugate according to claim 3, wherein in the single-chain antibody a light chain and a heavy chain of the anti-human transferrin receptor antibody are linked via a linker sequence.

15 5. The conjugate according to claim 4, wherein the light chain and the heavy chain of the anti-human transferrin receptor antibody are linked on the C-terminal side of the light chain via the linker sequence.

6. The conjugate according to claim 4, wherein the light chain and the heavy chain of the anti-human transferrin receptor antibody are linked on the C-terminal side of the heavy chain via the linker sequence.

20 7. The conjugate according to any one of claims 4 to 6, wherein the linker sequence comprises 8 to 50 amino acid residues.

25 8. The conjugate according to claim 7, wherein the linker sequence comprises an amino acid sequence selected from the group consisting of the amino acid sequence (Gly Ser), the amino acid sequence (Gly Gly Ser), the amino acid sequence (Gly Gly Gly), the amino acid sequence set forth as SEQ ID NO: 3, and the amino acid sequence set forth as

SEQ ID NO: 4.

9. The conjugate according to claim 8, wherein the linker sequence comprises 15 amino acid residues in which the amino acid sequence set forth as SEQ ID NO: 4 is consecutively repeated three times.

5 10. The conjugate according to any one of claims 1 to 9, wherein the anti-human transferrin receptor antibody comprises the amino acid sequence set forth as SEQ ID NO: 22 in the variable region of the light chain and the amino acid sequence set forth as SEQ ID NO: 23 in the variable region of the heavy chain.

10 11. The conjugate according to claim 10, wherein the amino acid sequence of the variable region of the light chain has an identity not lower than 80% to the amino acid sequence set forth as SEQ ID NO: 22, and the amino acid sequence of the variable region of the heavy chain has an identity not lower than 80% to the amino acid sequence set forth 15 as SEQ ID NO: 23.

12. The conjugate according to claim 10, wherein the amino acid sequence of the variable region of the light chain has an identity not lower than 90% to the amino acid sequence set forth as SEQ ID NO: 22, and the amino acid sequence of the variable region of the heavy chain has an identity not lower than 90% to the amino acid sequence set forth 20 as SEQ ID NO: 23.

13. The conjugate according to claim 10, wherein 1 to 10 amino acids constituting the variable region of the light chain are substituted with other amino acids relative to the amino acid sequence set forth as 25 SEQ ID NO: 22.

14. The conjugate according to claim 10, wherein 1 to 3 amino acids

constituting the variable region of the light chain are substituted with other amino acids relative to the amino acid sequence set forth as SEQ ID NO: 22.

15. The conjugate according to claim 10, wherein 1 to 10 amino acids constituting the variable region of the heavy chain are substituted with other amino acids relative to the amino acid sequence set forth as SEQ ID NO: 23.

10 16. The conjugate according to claim 10, wherein 1 to 3 amino acids constituting the variable region of the heavy chain are substituted with other amino acids relative to the amino acid sequence set forth as SEQ ID NO: 23.

15 17. The conjugate according to claim 10, wherein 1 to 10 amino acids constituting the variable region of the light chain are substituted with other amino acids relative to the amino acid sequence set forth as SEQ ID NO: 22, and 1 to 10 amino acids constituting the variable region of the heavy chain are substituted with other amino acids relative to the amino acid sequence set forth as SEQ ID NO: 23.

20 18. The conjugate according to claim 10, wherein 1 to 3 amino acids constituting the variable region of the light chain are substituted with other amino acids relative to the amino acid sequence set forth as SEQ ID NO: 22, and 1 to 3 amino acids constituting the variable region of the heavy chain are substituted with other amino acids relative to the amino acid sequence set forth as SEQ ID NO: 23.

25 19. The conjugate according to claim 10, wherein the anti-human transferrin receptor antibody comprises the light chain comprising an amino acid sequence set forth as SEQ ID NO: 24 and the heavy chain

comprising an amino acid sequence set forth as SEQ ID NO: 25.

20. The conjugate according to claim 19, wherein the amino acid sequence of the light chain has an identity not lower than 80% to the amino acid sequence set forth as SEQ ID NO: 24, and the amino acid sequence of the heavy chain has an identity not lower than 80% to the amino acid sequence set forth as SEQ ID NO: 25.

5 21. The conjugate according to claim 19, wherein the amino acid sequence of the light chain has an identity not lower than 90% to the amino acid sequence set forth as SEQ ID NO: 24, and the amino acid sequence of the heavy chain has an identity not lower than 90% to the amino acid sequence set forth as SEQ ID NO: 25.

10 22. The conjugate according to any of claims 1 to 21, wherein the agent is a peptide or a protein.

15 23. The conjugate according to claim 22, selected from the group consisting of (1) to (4) below:

(1) a conjugate in which the protein is linked to the C-terminal side of the light chain of the anti-human transferrin receptor antibody by a peptide bond,

20 (2) a conjugate in which the protein is linked to the N-terminal side of the light chain of the anti-human transferrin receptor antibody by a peptide bond,

(3) a conjugate in which the protein is linked to the C-terminal side of the heavy chain of the anti-human transferrin receptor antibody by a peptide bond, and

25 (4) a conjugate in which the protein is linked to the N-terminal side of the heavy chain of the anti-human transferrin receptor antibody

by a peptide bond.

24. The conjugate according to claim 23, wherein the protein is linked to the anti-human transferrin receptor antibody via a linker sequence.

5 25. The conjugate according to claim 24, wherein the linker sequence consists of 1 to 50 amino acid residues.

26. The conjugate according to claim 25, wherein the linker sequence comprises an amino acid sequence selected from the group consisting of a single glycine, a single serine, the amino acid sequence 10 (Gly Ser), the amino acid sequence (Gly Gly Ser), the amino acid sequence set forth as SEQ ID NO:3, the amino acid sequence set forth as SEQ ID NO:4, and the amino acid sequence consisting of 1 to 10 amino acid sequences thereof that are linked consecutively.

27. The conjugate according to any one of claims 1 to 28, wherein 15 the anti-human transferrin receptor antibody is a humanized anti-human transferrin receptor antibody.

28. The conjugate according to any one of claims 22 to 27, wherein the protein is a lysosomal enzyme.

29. The conjugate according to claim 28, wherein the lysosomal 20 enzyme is human α -galactosidase A.

30. The conjugate according to claim 29, wherein the light chain of the humanized anti-hTfR antibody comprises the amino acid sequence set forth as SEQ ID NO: 24, and wherein the heavy chain of the humanized anti-hTfR antibody is linked on its C-terminal side to the 25 human α -galactosidase A via a linker sequence (Gly Ser), and the whole linked heavy chain has the amino acid sequence set forth as SEQ ID

NO: 29.

31. The conjugate according to claim 28, wherein the lysosomal enzyme is human acid α -glucosidase.

32. The conjugate according to claim 31, wherein the light chain of the humanized anti-hTfR antibody comprises the amino acid sequence set forth as SEQ ID NO: 24, and wherein the heavy chain of the humanized anti-hTfR antibody is linked on its C-terminal side to the human acid α -glucosidase via a linker sequence (Gly Ser), and the whole linked heavy chain has the amino acid sequence set forth as SEQ ID NO: 27.

33. A pharmaceutical composition for improving a muscle function, comprising the conjugate according to any one of claims 1 to 32.

34. A pharmaceutical composition for ameliorating muscle dysfunction associated with lysosomal disease, comprising the conjugate according to claim 28.

35. A pharmaceutical composition for ameliorating muscle dysfunction associated with Fabry disease, comprising the conjugate according to claim 29 or 30.

36. A pharmaceutical composition for ameliorating muscle dysfunction associated with Pompe disease, comprising the conjugate according to claim 31 or 32.

37. The pharmaceutical composition according to any one of claims 33 to 36, wherein the muscle is skeletal muscle, cardiac muscle, or smooth muscle.

38. Use of the conjugate according to any one of claims 28 to 32 for the manufacture of a pharmaceutical composition for ameliorating

muscle dysfunction associated with lysosomal disease.

39. Use of the conjugate according to claim 29 or 30 for the manufacture of a pharmaceutical composition for ameliorating muscle dysfunction associated with Fabry disease.

5 40. Use of the conjugate according to claim 31 or 32 for the manufacture of a pharmaceutical composition for ameliorating muscle dysfunction associated with Pompe disease.

41. Use of the conjugate according to any one of claims 1 to 32 as a pharmaceutical composition for improving muscle function.

10 42. A method for delivering an agent to muscle, comprising a step of producing the agent as a conjugate in which the agent is linked to an anti-human transferrin receptor antibody and a step of administering the conjugate to an individual.

15 43. The method according to claim 41, wherein the conjugate is the conjugate according to any one of claims 1 to 32.

44. The method according to claim 42 or 43, further comprising a step of improving muscle function of the individual by administration of the conjugate.

20 45. The method according to any one of claims 42 to 44, wherein the individual has a muscle dysfunction.

46. The method according to any one of claims 42 to 44, wherein the individual has a muscle dysfunction associated with lysosomal disease.

25 47. The method of claim 46, wherein the lysosomal disease is Fabry disease or Pompe disease.

Fig.1

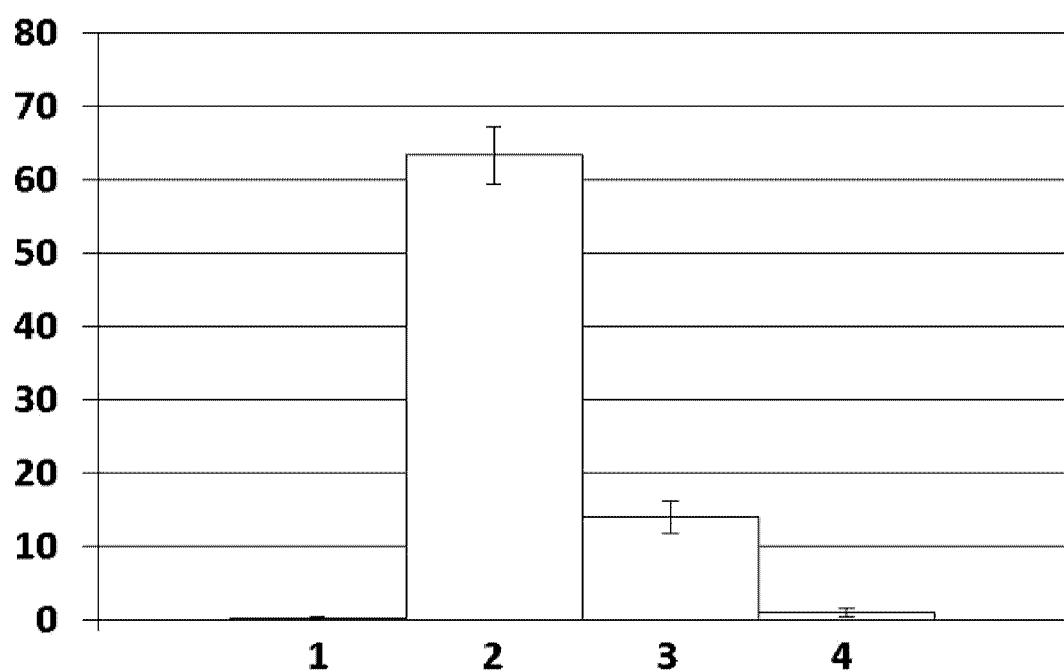


Fig.2

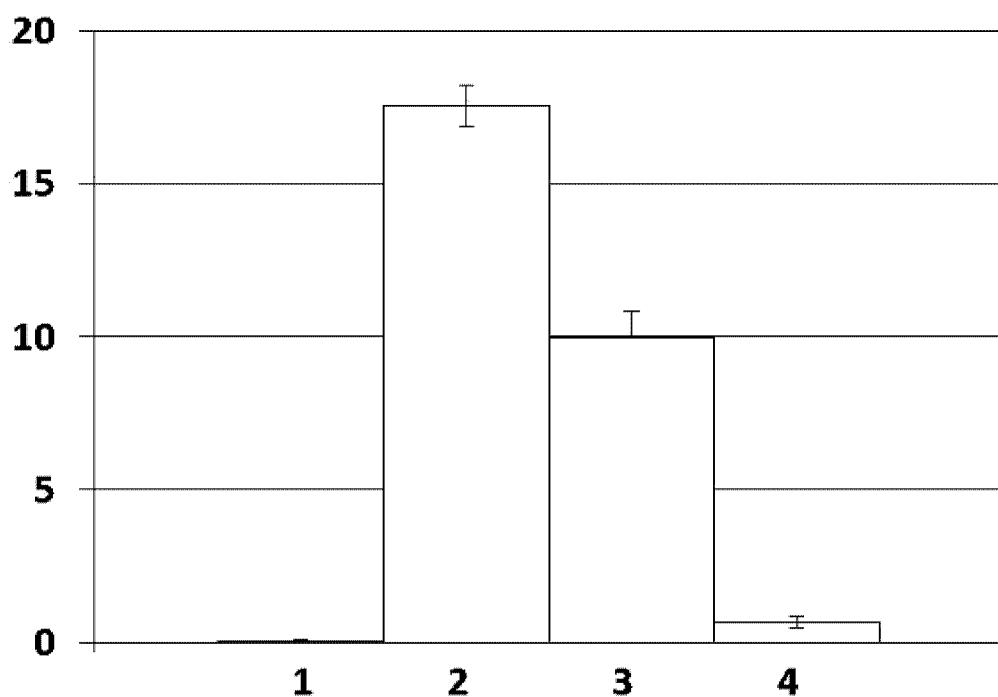


Fig.3

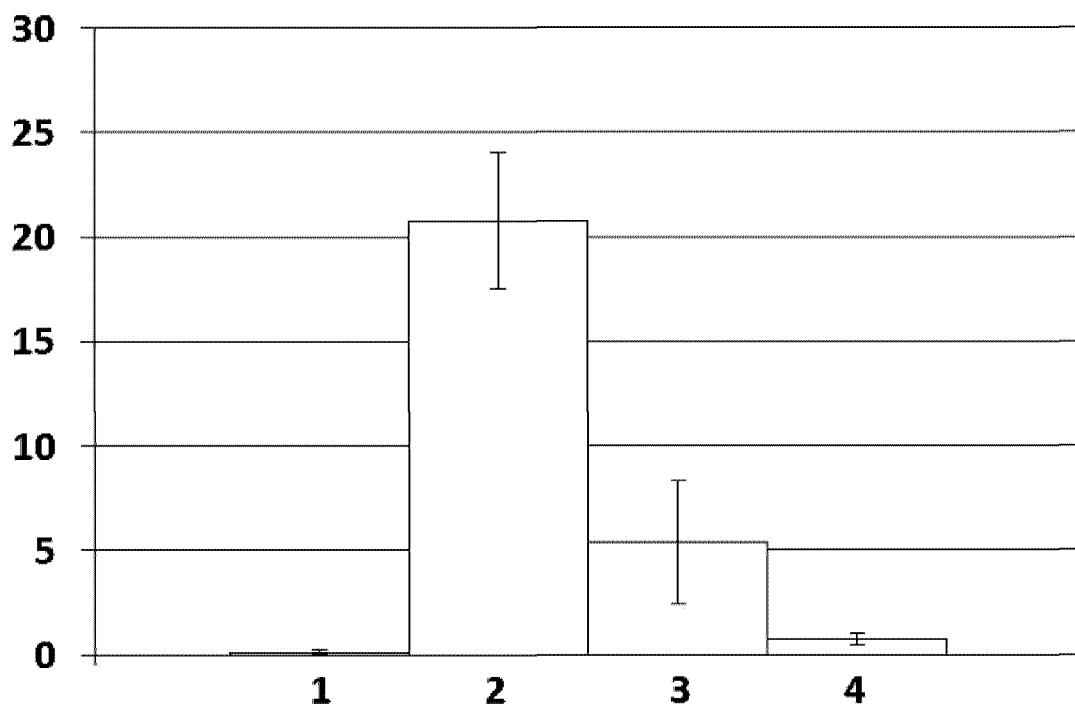


Fig.4

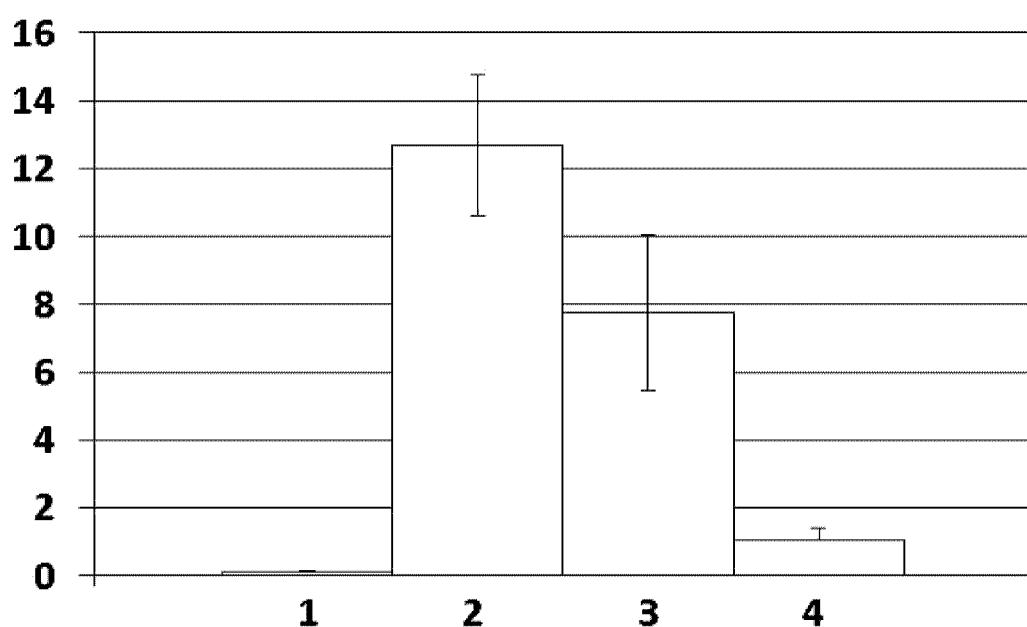


Fig.5

