GLYCOSYLATED IMMUNOGLOBULIN AND IMMUNOADHESIN COMPRISING THE SAME

Technical Field

The present invention relates to a glycosylated immunoglobulin and an immunoadhesin comprising the same. More particularly, the present invention relates to an immunoglobulin or a fragment thereof, which is additionally glycosylated by modification of a specific amino acid residue, and a glycosylated fusion protein formed as a result of linkage of the glycosylated immunoglobulin or a fragment thereof with at least one biologically active protein or a portion thereof.

Background Art

Immunoadhesins (or immunoglobulin fusion proteins) are antibody-like molecules resulting from the fusion of a fragment (e.g., Fc portion) of an immunoglobulin and a ligand-binding region of a receptor or an adhesive molecule. The typical immunoadhesins known in the art have the structure of an antibody in which the variable region, participating in antigen recognition, is replaced with a ligand-binding region of a receptor while retaining the Fc portion. For a long time, a large number of patents have described fusion proteins in which a specific region of a physiologically active protein is linked to an antibody (U.S. Pat. Nos. 5,521,288, 5,844,095, 6,046,310, 6,090,914, 6,100,383 and 6,225,448).

The immunoadhesin has the following advantages over a molecule not containing an immunoglobulin:

1) the fusion protein has increased total avidity to a ligand because it has bivalency in a dimer form;

2) the fusion protein is present in an undestroyed form in serum for a longer period of time by virtue of increased molecular stability;

3) effector cells are activated by the Fc (Fragment crystallizable) portion of the immunoglobulin heavy chain; and

4) the fusion protein is isolated and purified by a convenient method, for example, using protein A.

For example, in the case of tumor necrosis factor (hereinafter, referred to simply as "TNF") as a cytokine, to suppress TNF-dependent inflammation responses, tumor necrosis factor receptor (hereinafter, referred to simply as "TNFR") may be used as described in PCT Publication Nos. WO92/16221 and WO95/34326, or a TNFR-immunoglobulin(Ig) fusion protein may be used as described in U.S. Pat. Nos 5,447,851 and PCT Publication No. WO94/06476. According to numerous reports, TNFR-Ig fusion proteins have much higher affinity to TNF than the native monomer form or Ig-non-fused form of TNFR (Lesslauer W. et al. Eur. J. Immunol., 1991, vol.21, p.2883; Ashkenazi A. et al. PNAS USA, 1991, vol.88, p.10535; Peppel K. et al. J. Exp. Med., 1991, vol.174, p.1483; Mohler K.M. et al. J. Immunol., 1993, vol.151, p.1548).

With respect to the inhibition of TNF action or the control of immune responses using an Ig fusion protein, a multivalent or multimerized form of the extracellular domain as a functional domain of TNF receptors, CD2 and CTLA4 in an Ig fusion construct is expected to improve the efficacy of the fusion construct. When a monomeric fusion protein (heavy chain fusion protein) of the TNF receptor extracellular domain and the Ig heavy chain is expressed in a cell line simultaneously with another monomeric fusion protein (light chain fusion protein) of the TNF receptor extracellular domain and the Ig light chain, a dimeric fusion protein is produced by the interaction between the heavy chain and the light chain. The dimeric fusion protein has two effective domains arranged in parallel like the in vivo form, and has remarkably increased efficacy in comparison with monomeric fusion constructs (Scallon B.J. et al. Cytokine, 1995, vol.7, p.759).

However, such an Ig fusion protein in a dimeric form is difficult to industrialize due to the following problems: two genes which are individually fused to the Ig heavy and light chains should be co-introduced into a host cell; when two different fusion proteins are simultaneously expressed in a single cell, their yields greatly decrease; and because all expressed heavy chain fusion proteins and light chain fusion proteins do not participate in the formation of dimers, dimers that fuse a heavy chain fusion protein and a light chain fusion protien are technically difficult to isolate from a mixture with the monomeric heavy chain fusion proteins or light chain fusion proteins.

In this regard, the present inventors constructed a concatameric protein in which a C-terminus of the soluble domain of a biologically active protein is linked to an N-terminus of the soluble domain of an identical or different biologically active protein, using recombinant DNA technology. Also, the present inventors prepared a DNA construct encoding a dimeric protein in which two molecules of a monomeric protein, in which a concatamer of a protein participating in an immune response is linked to the hinge region of an immunoglobulin Fc fragment, are disulfide-bonded at the hinge region, and produced a concatamer-linked dimeric fusion protein using recombinant DNA technology based on the DNA construct.

As described above, attempts have been made to improve the efficacy and preparation method of immunoglobulin fusion proteins, but almost all efforts have been unable to increase the stability of the immunoglobulin fusion proteins. In this regard, as disclosed in Korean Pat. Application No. 2002-0045921, the present inventors developed a method of increasing protein stability by adding a glycosylation motif to a conjunction region between a functional domain of a protein and an immunoglobulin Fc region. However, when an immunoadhesin is glycosylated near a functional domain, the protein is not folded correctly or has reduced function.

Disclosure of the Invention

In this regard, the present inventors constructed a glycosylated fusion protein by introducing an additional glycosylation motif into an immunoglobulin, particularly an Fc portion, of an immunoglobulin fusion protein, and found that the glycosylated fusion protein acts *in vivo* for a longer period of time than the form not containing a glycosylation motif, thereby leading to the present invention.

Thus, in one aspect, the present invention provides a glycosylated immunoglobulin or a fragment thereof, in which an immunoglobulin variant comprising one or more amino acid modifications selected from the group consisting of M160N, A195N, T243N, E265N, Y299T, F331T and Q346N, is additionally glycosylated.

In another aspect, the present invention provides a DNA encoding a glycosylated immunoglobulin or a fragment thereof, in which an immunoglobulin variant, comprising one or more amino acid modifications selected from the group consisting of M160N, A195N, T243N, E265N, Y299T, F331T and Q346N, is additionally glycosylated.

In a further aspect, the present invention provides a glycosylated fusion protein formed as a result of linkage of (a) a glycosylated immunoglobulin or a fragment thereof, in which an immunoglobulin variant having a modified amino acid sequence forming one or more Asn-X-Ser/Thr sequences is additionally glycosylated, with (b) at least one biologically active protein or a portion thereof.

In yet another aspect, the present invention provides a DNA molecule encoding a glycosylated fusion protein, which is formed as a result of linkage of (a) a glycosylated immunoglobulin or a fragment thereof, in which an immunoglobulin variant having a modified amino acid sequence forming one or more Asn-X-Ser/Thr sequences is additionally glycosylated, with (b) at least one biologically active protein or a portion thereof.

In still another aspect, the present invention provides a recombinant expression vector comprising a DNA molecule encoding a glycosylated fusion protein, which is formed as a result of linkage of (a) a glycosylated immunoglobulin or a fragment thereof, in which an immunoglobulin variant having a modified amino acid sequence forming one or more Asn-X-Ser/Thr sequences is additionally glycosylated, with (b) at least one biologically active protein or a portion thereof.

In still another aspect, the present invention provides a host cell transfected or transformed with a recombinant expression vector comprising a DNA molecule encoding a glycosylated fusion protein, which is formed as a result of linkage of (a) a glycosylated immunoglobulin or a fragment thereof, in which an immunoglobulin variant having a modified amino acid sequence forming one or more Asn-X-Ser/Thr sequences is additionally glycosylated, with (b) at least one biologically active protein or a portion thereof.

In still another aspect, the present invention provides a method of preparing a glycosylated fusion protein, comprising culturing a host cell transfected or transformed with a recombinant expression vector comprising a DNA molecule encoding a glycosylated fusion protein, which is formed as a result of linkage of (a) a glycosylated immunoglobulin or a fragment thereof, in which an immunoglobulin variant having a modified amino acid sequence forming one or more Asn-X-Ser/Thr sequences is additionally glycosylated, with (b) at least one biologically active protein or a portion thereof; and isolating the glycosylated fusion protein from the culture.

In still another aspect, the present invention provides a pharmaceutical composition comprising a glycosylated fusion protein, which is formed as a result of linkage of (a) a glycosylated immunoglobulin or a fragment thereof, in which an immunoglobulin variant having a modified amino acid sequence forming one or more Asn-X-Ser/Thr sequences is additionally glycosylated, with (b) at least one biologically active protein or a portion thereof.

Brief Description of the Drawings

The above and other objects, features, and other advantages of the present invention will be more clearly understood from the following detailed description taken in conjunction with the accompanying drawings, in which:

FIG. 1 shows glycosylation sites of immunoglobulins according to the present invention;

FIG. 2 is a graph showing expression levels of glycosylated CTLA4-IgG fusion proteins according to the present invention;

FIG. 3 is a graph showing the results of Western blotting of glycosylated CTLA4-IgG fusion proteins according to the present invention; and

FIG. 4 is a graph showing changes over time in serum levels of glycosylated CTLA4-IgG fusion proteins according to the present invention, in mice intraperitoneally injected with the fusion proteins.

Best Mode for Carrying Out the Invention

Single capital letters representing amino acids, as used herein, represent the following amino acids according to the standard abbreviations defined by the International Union of Biochemistry:

A: Alanine; B: Asparagine or Asparatic acid;

C: Cysteine; D: Asparatic acid; E: Glutamic acid;

F: Phenylalanine; G: Glycine; H: Histidine;

I: Isoleucine; K: Lysine; L: Leucine; M: Methionine;

N: Aspargine; P: Proline; Q: Glutamine; R: Arginine;

S: Serine; T: Threonine; V: Valine; W: Tryptophan;

Y: Tyrosine; and Z: Glutamine or Glutamic acid.

The designation "(one capital for an amino acid)(amino acid position)(one capital for another amino acid)", as used herein, means that the former amino acid is substituted with the latter amino acid at the designated amino acid position of a given protein. For example, M179N indicates that the methionine residue at the 179th position of a given protein (i.e., IgG) is replaced with asparagine. The amino acid position is numbered from the N-terminus of a mature wild-type protein.

The term "glycosylation" means a process by which proteins produced by eukaryotic cells as host cells are modified by the attachment of sugar chains. The attachment of sugar chains is known to affect properties of proteins as well as in vivo stability and functionality of the proteins. There are two types of glycosylation. O-linked glycosylation links an oligosaccharide chain to a serine and/or threonine residue. N-linked glycosylation links an oligosaccharide chain to an asparagine residue. In particular, N-linked glycosylation occurs in the specific amino acid sequence, Asn-X-Ser/Thr (X is any amino acid excluding proline).

In the present invention, a DNA sequence encoding an immunoglobulin or a fragment thereof is mutated at one or more nucleotides to form an additional glycosylation site at which O-linked or N-linked glycosylation occurs, and the mutated DNA is expressed in a host cell to allow spontaneous glycosylation. In one aspect, a glycosylated immunoglobulin or a fragment thereof according to the present invention is constructed by mutating a DNA sequence encoding an immunoglobulin or a fragment thereof to add and/or increase an Asn-X-Ser/Thr sequence (glycosylation motif) in which N-linked glycosylation occurs.

The "immunoglobulins", which are modified to possess a glycosylation motif in the present invention, are protein molecules that are produced in B cells and serve as antigen receptors specifically recognizing a wide variety of antigens. The molecules have a Y-shaped structure consisting of two identical light chains (L chains) and two identical heavy chains (H chains), in which the four chains are held together by a number of disulfide bonds, including the disulfide bridge between the H chains at the

hinge region. The L and H chains comprise variable and constant regions. According to features of the constant regions of H chains, immunoglobulins (Ig) are classified into five isotypes, A (IgA), D (IgD), E (IgE), G (IgG) and M (IgM). The five subtypes possess unique structural and biological properties. These immunoglobulins may all be modified according to the present invention.

Since an immunoadhesin generally contains a fragment of an immunoglobulin, namely, the Fc portion, a glycosylation motif in the present invention is preferably introduced into the Fc portion of an immunoglobulin. The term "Fc portion of an immunoglobulin", as used herein, refers to a fragment having no antigen-binding activity and being easily crystallized, which comprises a hinge region and CH2 and CH3 domains, and a portion responsible for binding of an antibody to effector materials and cells.

In the present invention, a glycosylation motif is preferably created by modifying one or more amino acid residues at positions 160, 195, 243, 265, 299, 331 and 346 of an immunoglobulin (all of these amino acid residues are present at the Fc portion of an immunoglobulin). Thus, in one aspect, the present invention provides a glycosylated immunoglobulin or a fragment thereof, which comprises one or more amino acid modifications selected from the group consisting of M160N, A195N, T243N, E265N, Y299T, F331T and Q346N, and a gene encoding the same. In more detail, a glycosylated immunoglobulin or a fragment thereof listed in Table 1 is provided, which contains combinations of one or more of the aforementioned amino acid modifications.

TABLE 1

Glycosylated immunoglobulins or fragments thereof according to the present invention

Name	Amino acid modification	SEQ ID NO	
		DNA	Protein
Ig-G1	M160N	16	17
Ig-G2	E265N; Y299T	18	19
Ig-G3	Y299T; F331T	20	21
Ig-G4	M160N; E265N; Y299T	22	23
Ig-G5	M160N; E265N; Y299T; F331T	24	25
Ig-G6	M160N; A195N; E265N; Y299T; F331T	26	27
Ig-G7	M160N; A195N; T243N; E265N; Y299T; F331T	28	29
Ig-G8	M160N; A195N; T243N; E265N; Y299T; F331T; Q346N	30	31

In another aspect, the present invention provides a glycosylated fusion protein formed as a result of linkage of (a) a glycosylated immunoglobulin or a fragment thereof, in which an immunoglobulin variant having a modified amino acid sequence forming one or more Asn-X-Ser/Thr sequences is additionally glycosylated, with (b) at least one biologically active protein or a portion thereof. In a preferred aspect, the fragment of an immunoglobulin includes an Fc portion, and the portion of a biologically active protein includes a soluble extracellular domain.

In one aspect, the glycosylated fusion protein has a monomer structure in which a single polypeptide is formed as a result of linkage of (a) a glycosylated immunoglobulin or a fragment thereof, in which an immunoglobulin variant having a modified amino acid sequence forming one or more Asn-X-Ser/Thr sequences is additionally glycosylated, with (b) at least one biologically active protein or a portion thereof. The portion of a biologically active protein preferably includes a soluble extracellular domain of the biologically active protein. Two molecules of such a monomeric glycosylated fusion protein may be linked by a disulfide bond at the hinge region to form a dimer structure.

In another aspect, the glycosylated fusion protein has a monomer structure in which a single polypeptide is formed as a result of linkage, in a concatameric form, of (a) a glycosylated

immunoglobulin or a fragment thereof, in which an immunoglobulin variant having a modified amino acid sequence forming one or more Asn-X-Ser/Thr sequences is additionally glycosylated, with (b) a first biologically active protein or a portion thereof, and (c) a second biologically active protein or a portion thereof. The first and second biologically active proteins may be identical or different. The portion of a biologically active protein preferably includes a soluble extracellular domain of the biologically active protein. Two molecules of such a monomeric glycosylated fusion protein may be linked by a disulfide bond at the hinge region to form a dimer structure.

In a preferred aspect of the glycosylated fusion protein according to the present invention, the immunoglobulin variant comprises one or more amino acid modifications selected from the group consisting of M160N, A195N, T243N, E265N, Y299T, F331T and Q346N and is glycosylated. In a more preferred aspect, the immunoglobulin variant comprises any one of the amino acid sequences of SEQ ID NO: 16 to SEQ ID NO: 23, and in the most preferred aspect, the amino acid sequence of SEQ ID NO: 19.

The term "biologically active protein", as used herein, refers to a protein, peptide or polypeptide having generally physiological or pharmaceutical activities, which retains a part of its native activities after forming an immunoadhesin. The term "biological activity", as used herein, is not limited in meaning to physiological or pharmaceutical activities. For example, some immunoadhesins, such as those containing an enzyme, can catalyze a reaction in an organic solvent.

Non-limiting examples of the protein, peptide or polypeptide include hemoglobin, serum proteins (e.g., blood factors including factor VII, VIII and factor IX), immunoglobulin, cytokines (e.g., interleukin), α-, β- and γ-interferons, colony-stimulating factors (e.g., G-CSF and GM-CSF), platelet-derived growth factor (PDGF), and phospholipase activating proteins (PLAPs). Other typical biological or therapeutic proteins include insulin, plant proteins (e.g., lectin and ricin), tumor necrosis factor (TNF) and its mutant alleles, growth factors (e.g., tissue growth factors and endothelial growth factors such as TGFα or TGFβ), hormones (e.g., follicle-stimulating hormone, thyroid-stimulating

hormone, antidiuretic hormone, pigment-concentrating or dispersing hormones and parathyroid hormone, luteinizing hormone-releasing hormone and its derivatives), calcitonin, calcitonin gene related peptide (CGRP), synthetic enkephalin, somatomedin, erythropoietin, hypothalamus releasing factors, prolactin, chronic gonadotrophin, tissue plasminogen-activating agents, growth hormone-releasing peptide (GHRP), and thymic humoral factor (THF). Some proteins such as interleukin, interferon or colony-stimulating factor may be produced in a non-glycosylated form by using DNA recombinant techniques. The non-glycosylated proteins may be useful as biologically active materials in the present invention.

In addition, the biologically active materials useful in the present invention include any part of a polypeptide, which has bioactivity in vivo. Examples of the biologically active materials include peptides or polypeptides, fragments of an antibody, single chain-binding proteins (see, U.S. Pat. No. 4,946,778), binding molecules including fusion polypeptides of antibodies or their fragments, polyclonal antibodies, monoclonal antibodies, and catalytic antibodies. Other examples of the biologically active materials include allergen proteins, such as ragweed, antigen E, honeybee venom, or allergen of mites.

In addition, the biologically active material useful in the present invention includes enzymes. Examples of the enzymes include carbohydrate-specific enzymes, proteinases, oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases. In detail, Non-limiting examples of the enzymes include asparaginase, arginase, arginine deaminase, adenosine deaminase, peroxide dismutase, endotoxinase, catalase, chymotrypsin, lipase, uricase, adenosine dephosphatase, tyrosinase, and bilirubin oxidase. Examples of the carbohydrate-specific enzymes include glucose oxidase, glucodase, galactosidase, glucocerebrosidase, and glucouronidase.

The term "soluble extracellular domain", as used herein, refers to a portion exposed to the extracellular region of an integral membrane protein penetrating the cell membrane comprising phospholipid, wherein the integral membrane protein contains one or more transmembrane domains

made up predominantly of hydrophobic amino acids. Such an extracellular domain mainly comprises hydrophilic amino acids, which are typically positioned at the surface of a folded structure of a protein, and thus is soluble in an aqueous environment. Of most cell surface receptor proteins, extracellular domains serve to bind specific ligands, while intracellular domains play an important role in signal transduction.

In one aspect, the glycosylated fusion protein according to the present invention may be prepared by preparing a DNA sequence encoding an immunoglobulin or a fragment thereof which is modified to contain a glycosylation site and linking thereto another DNA sequence encoding a biologically active protein or a portion thereof. In another aspect, the glycosylated fusion protein may be prepared by primarily preparing a DNA sequence (fusion gene) that encodes both an immunoglobulin or a fragment thereof and a biologically active protein or a portion thereof, and mutating the fusion gene to allow the immunoglobulin or the fragment thereof to be glycosylated. The two preparation methods differ from each other only in terms of a DNA sequence serving as a template and are basically identical to preparation methods, known in the art, of a DNA sequence encoding a protein variant. Thus, hereinafter, the present invention intends to focus on a modification method of an immunoglobulin or a fragment thereof into which a glycosylation motif is substantially introduced.

A DNA sequence encoding the glycosylated immunoglobulin or the fragment thereof according to the present invention may be prepared according to various methods known in the art. These methods include, but are not limited to, oligonucleotide-mediated mutagenesis and cassette mutagenesis.

In particular, the DNA sequence encoding the glycosylated immunoglobulin or the fragment thereof according to the present invention is preferably prepared by oligonucleotide-mediated mutagenesis. This technique is well known in the art, and described by Zoller M. et al. (Zoller M. et al. Nuc. Ac. Res. USA, 1982, vol.10, pp.6487-6500). In brief, the DNA sequence encoding the glycosylated immunoglobulin or the fragment thereof may be prepared by hybridizing a template DNA

(e.g., a plasmid carrying DNA encoding a non-modified or native immunoglobulin or a fragment thereof) with an oligonucleotide coding for a desired modification. After hybridization, a second complete strand complementary to the DNA template may be synthesized by DNA polymerase, and the second strand may code for the desired modification.

Typically, oligonucleotides used in the aforementioned methods are composed of about 25 nucleotides. Shorter oligonucleotides can be employed, but optimal oligonucleotides, at both left and right regions of modified codons, contain 12 to 15 nucleotides complementary to a template. These oligonucleotides can effectively hybridize with a template DNA. These oligonucleotides may be synthesized by the techniques known in the art (Crea et al. Proc. Natl. Acad. Sci. USA, 1978, vol.75, p.5765).

In one aspect, the present invention provides a DNA sequence encoding an immunoglobulin or a fragment thereof, which carries one amino acid modification (IgG in Table 1). This DNA sequence may be prepared by performing PCR using DNA encoding an immunoglobulin or its fragment as a template and modification-encoding synthetic oligonucleotides as primers. Primers hybridize with their complementary single-stranded DNA produced by denaturation of a double-stranded DNA template during heating. DNA polymerase adds nucleotides to the 3′-OH of the modification-encoded primer one by one in a manner complementary to a template in the 5′ to 3′ direction. The newly synthesized strand incorporates the modification-encoded primer, thus yielding a gene encoding a desired modification. The newly synthesized strand is used as a template DNA in the extension step of PCR, resulting in amplification of a gene encoding the modification.

In another aspect, the present invention provides a DNA sequence encoding an immunoglobulin or a fragment thereof, which carries two or more amino acid modifications. When two or more amino acids to be modified are spaced close to each other on a polypeptide, all desired modifications are encoded in one oligonucleotide and thus simultaneously achieved. Therefore, a mutated immunoglobulin or a fragment thereof having two or more amino acid modifications may be

prepared by the same method used to prepare the mutated immunoglobulin or fragment thereof carrying one nucleotide modification, excepting for the use of oligonucleotides containing two or more amino acid modifications as primers.

When two or more amino acids to be modified are spaced far apart (in the case that over 10 amino acids are present between two amino acids to be modified), all desired modifications cannot be encoded in one oligonucleotide. Thus, different methods should be introduced. One method is to prepare individual oligonucleotides for each amino acid modification. When the oligonucleotides are annealed simultaneously to a single-stranded template DNA, a newly synthesized secondary single-stranded DNA encodes all of the desired amino acid modifications. Another approach used in the present invention includes two mutagenesis experiments. In the primary mutagenesis, using natural DNA as a template, one oligonucleotide containing one desired amino acid modification is annealed to the template, and thus heteroduplex DNA is produced. In the secondary mutagenesis, the heteroduplex DNA is used as a template. The template already carries at least one modification. When one oligonucleotide having an additional amino acid modification is annealed to the template, the resulting DNA encodes both of the primary and secondary modifications.

The cassette mutagenesis is also a preferred method for the preparation of DNA encoding the glycosylated immunoglobulin or fragment thereof according to the present invention. This method is based on the technique described by Well J. et al. (Well J. et al. Biochem., 1990, vol.29, pp.8509-8517). A starting material is a plasmid (or another vector) containing a gene encoding an immunoglobulin or a fragment thereof to be modified. The cassette mutagenesis is preferably used when a specific restriction enzyme site is present only at a position to be modified. However, this is not essential. If such a restriction enzyme site does not exist, it can be introduced into an appropriate position of a gene encoding an immunoglobulin or a fragment thereof by oligonucleotide-mediated mutagenesis. After a restriction enzyme site is introduced into the plasmid, the plasmid is linearized by treatment with the restriction enzyme. A double-stranded oligonucleotide having a DNA sequence that contains a

desired mutation and is located between restriction enzyme sites may be synthesized using a common method. The two strands are individually synthesized and hybridized using a common technique. Such a double-stranded oligonucleotide is typically designated "a cassette". The cassette should be prepared in the form of possessing 3′- and 5′-ends that are compatible with both ends of the linearized plasmid and may be thus directly conjugated to the plasmid. The plasmid comes to contain a DNA encoding a desired glycosylated immunoglobulin or a fragment thereof through the aforementioned procedure.

In addition, the preparation of a DNA sequence encoding a glycosylated immunoglobulin or a fragment thereof according to the present invention may be achieved by a chemical method. In particular, such a DNA sequence may be synthesized by a chemical method using an oligonucleotide synthesizer. An oligonucleotide is made based on an amino acid sequence of an glycosylated immunoglobulin or a fragment thereof, and preferably by selecting a preferable codon using a host cell producing an glycosylated immunoglobulin or a fragment thereof.

With respect to a DNA sequence encoding a glycosylated immunoglobulin or a fragment thereof according to the present invention, the degeneracy in the genetic code, which means that one amino acid is specified by more than one codon, is well known in the art. Thus, there is a plurality of DNA sequences with degeneracy encoding a glycosylated immunoglobulin or a fragment thereof according to the present invention, and they all fall into the scope of the present invention.

Alternatively, the glycosylated fusion protein according to the present invention may be prepared as follows. A DNA sequence encoding the fusion protein (hereinafter, referred to as "fusion gene") is prepared, and is inserted into a vector including one or more expression control sequences regulating the expression of the fusion gene by being operably linked to the fusion gene. Then, a host is transformed or transfected with the resulting recombinant expression vector. The resulting transformant or transfectant is cultured in a suitable medium under conditions suitable for the expression

of the fusion gene. A substantially pure glycosylated fusion protein coded by the fusion gene is recovered from the resulting culture.

The term "vector", as used herein, means a DNA molecule that serves as a vehicle capable of stably carrying exogeneous genes into host cells. To be useful in application, a vector should be replicable, have a system for introducing itself into a host cell, and possess selectable markers.

In addition, the term "recombinant expression vector", as used herein, refers to a circular DNA molecule carrying exogeneous genes operably linked thereto to be expressed in a host cell. When introduced into a host cell, the recombinant expression vector has the ability to replicate regardless of host chromosomal DNA at a high copy number and to produce heterogeneous DNA. As generally known in the art, in order to increase the expression level of a transfected gene in a host cell, the gene should be operably linked to transcription and translation regulatory sequences functional in the host cell selected as an expression system. Preferably, the expression regulation sequences and the exogeneous genes may be carried in a single expression vector containing selectable markers and a replication origin. In the case that eukaryotic cells are used as an expression system, the expression vector should further comprise expression markers useful in the eukaryotic host cells.

In order to express the DNA sequence (i.e. fusion gene) encoding the glycosylated fusion protein according to the present invention, various expression vectors may be employed. Preferably, since an immunoglobulin or a fragment thereof should be glycosylated, expression vectors suitable for eukaryotic host cells should be used. Expression vectors useful for eukaryotic host cells contain expression control sequences derived from, for example, SV40, bovine papilloma virus, adenovirus and cytomegalovirus. In detail, examples of the vectors include pCDNA3.1(+)/Hyg (Invitrogen, Carlsbad, Calif., USA) and pCI-neo (Stratagen, La Jolla, Calif., USA). Expression vectors useful for yeasts include 2μ plasmid and its isoforms, POT1 vector (U.S. Pat. No. 4,931,373) and pPICZ A, B, or C (Invitrogen). Expression vectors useful for insect cells include pVL 941, pBluebac 4.5 and pMelbac (Invitrogen). However, the present invention is not limited to these examples.

The term "expression control sequences", as used herein, refers to nucleotide sequences necessary or advantageous for expression of the fusion gene according to the present invention. Each control sequence may be native or foreign to the fusion gene. Non-limiting examples of the expression control sequences include leader sequences, polyadenylation sequences, propeptide sequences, promoters, enhancers or upstream activating sequences, signal peptide sequences, and transcription terminators.

In order to express the fusion gene of the present invention, any of the various expression control sequences may be inserted into the expression vectors used in the present invention. Examples of expression control sequences suitable for directing protein expression in mammalian cells include SV40 and early and late promoters of adenovirus, MT-1 (metallothionein gene) promoter, human cytomegalovirus immediate-early gene promoter (CMV), Rouse sarcoma virus (RSV) promoter, and human ubiquitin C (UbC) promoter. In addition, to improve expression levels in mammalian cells, a synthetic intron may be inserted into the 5'-untranslated region of the fusion gene. Examples of expression control sequences suitable for directing protein expression in insect cells include polyhedrin promoter, P10 promoter, baculovirus 39K delayed-early gene promoter and SV40 polyadenylation sequence. Examples of expression control sequences suitable for directing protein expression in yeasts include the promoter of the yeast α-mating system, yeast triose-phosphate isomerase (TPI) promoter and ADH2-4c promoter. Examples of expression control sequences suitable for directing protein expression in fungal cells include ADH3 promoter and terminators.

The term "operably linked" refers to a state in which the fusion gene of the present invention is arranged with such a control sequence in a functional relationship. That is, a gene and control sequences are linked in such a manner that expression of the gene is induced when a suitable molecule (e.g., transcription-activating protein) binds to the control sequence(s). For example, when a pre-sequence or secretory leader facilitates secretion of a mature protein, it is referred to as operably linked to the coding sequence of the protein. A promoter is operably linked with a coding sequence when it

regulates transcription of the coding sequence. A ribosome-binding site is operably linked to a coding sequence when it is present at a position allowing translation of the coding sequence. Typically, the term "operably linked" means that linked nucleotide sequences are in contact with each other. In the case of a secretory leader sequence, the term means that it contacts a coding sequence and is present within a reading frame of the coding sequence. However, an enhancer need not necessarily contact a coding sequence. Linkage of the nucleotide sequences may be achieved by ligation at convenient restriction enzyme recognition sites. In the absence of restriction enzyme recognition sites, oligonucleotide adaptors or linkers may be used, which are synthesized by the conventional methods.

On the other hand, host cells having high introduction efficiency of foreign DNA and having high expression levels of an introduced DNA may be used. In particular, as a host cell, a eukaryotic cell capable of glycosylating the fusion protein of the present invention should be used. Examples of suitable yeast host cells include strains of *Saccharomyces* and *Hansenula*. Examples of suitable fungal host cells include *Tricoderma*, *Fusarium* and *Aspergillus* species. Examples of suitable insect host cells include *Lepidoptora* cell lines such as Sf9 or Sf21. Examples of suitable mammalian host cells include CHO cell lines, COS cell lines such as COS1 or COS7, animal cell lines such as BHK cell line or mouse cells, and tissue-cultured plant cells and human cells.

The fusion gene of the present invention or a recombinant expression vector comprising the same may be introduced into a host cell by the methods described in basic experimental guide books (e.g., Davis et al., Basic Methods in Molecular Biology (1986). The preferred methods for this introduction into a host cell include, for example, calcium phosphate transfection, DEAE-dextran mediated transfection, microinjection, cationic lipid-mediated transfection, electroporation, viral transduction, scrape loading, ballistic introduction, and infection.

In the preparation method of the present invention, the host cells are cultivated in a nutrient medium suitable for production of a polypeptide using methods known in the art. For example, the cells may be cultivated by shake flask cultivation, small-scale or large-scale fermentation in laboratory

or industrial fermentors performed in a suitable medium and under conditions allowing the polypeptide to be expressed and/or isolated. The cultivation takes place in a suitable nutrient medium containing carbon and nitrogen sources and inorganic salts, using procedures known in the art. Suitable media are commercially available from commercial suppliers and may be prepared according to published compositions (for example, the catalog of American Type Culture Collection). If the fusion protein is secreted into the nutrient medium, it can be recovered directly from the medium. If the fusion protein is not secreted, it can be recovered from cell lysates.

The glycosylated fusion protein of the present invention may be recovered using any one of a number of methods for isolating a polypeptide, which are known in the art. For example, the polypeptide may be recovered from the nutrient medium by conventional procedures including, but not limited to, centrifugation, filtration, extraction, spray drying, evaporation, or precipitation. Further, the polypeptide may be purified by a variety of procedures known in the art including, but not limited to, chromatography (e.g., ion exchange, affinity, hydrophobicity, and size exclusion), electrophoresis, differential solubility (e.g., ammonium sulfate precipitation), SDS-PAGE, or extraction.

In a further aspect, the present invention provides a pharmaceutical composition comprising the glycosylated fusion protein according to the present invention.

The term "treatment", as used herein, refers to a perfect cure, suppression or alleviation of diseases or disorders. Therefore, the term "therapeutically effective amount", as used herein, means an amount sufficient to achieve the above pharmaceutical effect. In the present invention, the therapeutically effective amount may vary according to the formulation methods, administration modes, patient's age, weight and gender, severity of the illness, diets, administration duration, administration routes, excretion rates, and response sensitivity. Those skilled in the art may readily determine and prescribe a dosage capable of achieving a desired treatment.

In addition, it will be apparent to those skilled in the art that the diseases to be treated by the pharmaceutical composition of the present invention may be varied by varying the type of protein. A

glycosylated CTLA4-Ig fusion protein as an embodiment of the present invention is applicable to diseases against which it displays therapeutic effects by inhibiting the action of T-cells, for example, autoimmune diseases such as arthritis or psoriasis, various organ transplants including bone marrow transplants, and varicose veins. Also, fusion proteins with receptors for various cancer-associated cell growth factors may be used in the treatment of cancer because they have improved therapeutic efficacy due to their effects of increasing serum levels of the receptors and blocking angiogenic factors.

The carrier used in the pharmaceutical composition of the present invention includes the commonly used carriers, adjuvants and vehicles, in the pharmaceutical field, which are as a whole called "pharmaceutically acceptable carriers". Non-limiting pharmaceutically acceptable carriers useful in the pharmaceutical composition of the present invention include ion exchange resin, alumina, aluminum stearate, lecithin, serum proteins (e.g., human serum albumin), buffering agents (e.g., sodium phosphate, glycine, sorbic acid, potassium sorbate, partial glyceride mixtures of saturated vegetable fatty acids), water, salts or electrolytes (e.g., protamine sulfate, disodium hydrophosphate, potassium hydrophoshate, sodium chloride, and zinc salts), colloidal silica, magnesium trisilicate, polyvinylpyrrolidone, cellulose-based substrates, polyethylene glycol, sodium carboxymethylcellulose, polyarylate, waxes, polyethylene-polyoxypropylene-block copolymers, polyethylene glycol, and wool fat.

The pharmaceutical composition of the present invention may be administered via any of the common routes, if it is able to reach a desired tissue. Therefore, the pharmaceutical composition of the present invention may be administered topically, parenterally, intraocularly, transdermally, intrarectally and intraluminally, and may be formulated into solutions, suspensions, and the like. The term "parenteral", as used herein, includes subcutaneous, intranasal, intravenous, intraperitoneal, intramuscular, intra-articular, intra-synovial, intrasternal, intracardial, intrathecal, intralesional and intracranial injection or infusion techniques.

In one aspect, the pharmaceutical composition of the present invention may be formulated as aqueous solutions for parenteral administration. Preferably, a suitable buffer solution, such as Hank's solution, Ringer's solution or physiologically buffered saline, may be employed. Aqueous injection suspensions may be supplemented with substances capable of increasing viscosity of the suspensions, which are exemplified by sodium carboxymethylcellulose, sorbitol and dextran. In addition, suspensions of the active components, such as oily injection suspension, include lipophilic solvents or carriers, which are exemplified by fatty oils such as sesame oil, and synthetic fatty acid esters such as ethyl oleate, triglycerides or liposomes. Polycationic non-lipid amino polymers may also be used as vehicles. Optionally, the suspensions may contain suitable stabilizers or drugs to increase the solubility of components and obtain high concentrations of the components.

The pharmaceutical composition of the present invention is preferably in the form of a sterile injectable preparation, such as a sterile injectable aqueous or oleaginous suspension. Such suspension may be formulated according to the methods known in the art, using suitable dispersing or wetting agents (e.g., Tween 80) and suspending agents. The sterile injectable preparations may also be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, such as a solution in 1,3-butanediol. The acceptable vehicles and solvents include mannitol, water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile fixed oils may conventionally be employed as a solvent or suspending medium. For this purpose, any bland fixed oil may be employed, including synthetic mono- or di-glycerides. In addition, fatty acids, such as oleic acid and glyceride derivatives thereof, may be used in the preparation of injectable preparations, like the pharmaceutically acceptable natural oils (e.g., olive oil or castor oil), and particularly, polyoxyethylated derivatives thereof.

The aforementioned aqueous composition is sterilized mainly by filtration using a filter to remove bacteria, mixing with disinfectants or in combination with radiation. The sterilized

composition can be hardened, for example, by freeze-drying to obtain a hardened product, and for practical use, the hardened composition is dissolved in sterilized water or a sterilized diluted solution.

In order to increase stability at room temperature, reduce the need for high-cost storage at low temperature, and prolong shelf-life, the pharmaceutical composition of the present invention may be lyophilized. A process for freeze-drying may comprise the steps of freezing, first drying and second drying. After freezing, the composition is heated under pressure to evaporate the water. At the second drying step, residual water is removed from the dry product.

The daily effective dosage of the pharmaceutical composition according to the present invention is typically about 10 μg to about 500 μg per kg body weight, preferably about 20 μg to about 300 μg per kg body weight, and most preferably about 50 μg to about 200 μg per kg body weight. It will be apparent to those skilled in the art that the specific amount of the pharmaceutical composition to be administered to a patient may vary depending on a plurality of factors, including, but not limited to, desired biological activity, the patient's symptoms and drug resistance.

A better understanding of the present invention may be obtained through the following examples. It will be apparent to those skilled in the art that the following examples are provided only to illustrate the present invention, and the scope of the present invention is not limited to the examples.

EXAMPLES

TABLE 2

Information on primers used in the preparation of CTLA4-Ig

Primer name and others	SEQ ID NO.	Description
oligo CTLA4-F-EcoRI	1	Containing the 5′ end of the soluble extracellular domain of CTLA4 and an EcoRI site
oligo CTLA4-R-Pst I	2	Containing the 3′ end of the soluble extracellular domain of CTLA4 and a PstI site
oligo IgG1-F-Pst I	3	Containing the 5′ end of the IgG hinge region and a PstI site
oligo IgG1-R-Xba I	4	Containing the 3′ end of IgG and an XbaI site
IgG-coding DNA	5	DNA encoding wild-type IgG
IgG protein	6	Wild-type IgG protein
CTLA4-IgG-coding DNA	7	DNA encoding a fusion protein in which IgG Fc is linked to the soluble extracellular domain of CTLA4
CTLA4-IgG protein	8	Fusion protein in which IgG Fc is linked to the soluble extracellular domain of CTLA4

EXAMPLE 1

A. Preparation of DNA fragment encoding soluble extracellular domain of CTLA4

A DNA fragment encoding a soluble extracellular domain of CTLA4 was prepared by PCR using a primer (SEQ ID NO: 1) having a recognition sequence of a restriction enzyme, EcoRI, and a coding sequence for a leader sequence of CTLA4, and another primer (SEQ ID NO: 2) having a PstI recognition sequence and an antisense sequence coding for a 3′ end of the soluble extracellular domain of CTLA4. A cDNA template in the PCR was prepared by reverse transcription polymerase chain reaction (RT-PCR) using mRNA extracted from mononuclear cells (T lymphocytes) of healthy adults. mRNA was isolated using a Tri-Reagent mRNA isolation kit (MRC, USA). First, 2×10^7 human T

lymphocytes were washed with phosphate buffered saline (PBS, pH 7.2) three times and lysed with 1 ml Tri-Reagent by repetitive pipetting. The cell lysate was mixed with 0.2 ml chloroform by vigorous shaking, allowed to stand at room temperature for 15 min, and centrifuged at 15,000 rpm at 4°C for 15 min. The supernatant was transferred to a 1.5-ml tube, mixed with 0.5 ml isopropanol, and centrifuged at 15,000 rpm at 4°C for 15 min. After the supernatant was discarded, the pellet was washed with 1 ml of triple-distilled water (TDW) treated with 75% ethanol-25% DEPC. The tube was inverted twice or three times and centrifuged at 15,000 rpm at 4°C for 15 min. After the supernatant was completely removed, the RNA pellet was air-dried to remove remaining ethanol and dissolved in 50 μl DEPC-treated TDW.

B. Preparation of DNA fragment encoding Fc region of IgG1

A DNA fragment encoding an Fc region of IgG1 was prepared by PCR using a primer (SEQ ID NO: 3) having a PstI recognition sequence and a sequence coding for a 5′ end of IgG1 Fc, and another primer (SEQ ID NO: 4) having an XbaI recognition sequence and an antisense sequence coding for a 3′ end of IgG1 Fc. A cDNA template in the PCR was prepared by RT-PCR using mRNA extracted from peripheral blood cells (B lymphocytes) of patients having a fever of unknown origin, who were in recovery. RT-PCR was carried out using the same reagents under the same conditions as in Example 1, part A.

C. Preparation of gene encoding non-glycosylated CTLA4-IgG

The DNA fragment encoding the soluble extracellular domain of CTLA4 and the DNA fragment encoding the Fc region of IgG1 were digested with PstI and ligated using T4 DNA ligase. The ligated DNA contained a leader sequence to facilitate protein secretion after expression. The fusion gene fragment thus produced was digested with EcoRI and XbaI and inserted into EcoRI/XbaI sites of pBluescript KS II(+) (Stratagene, USA), which is a commercially available cloning vector.

The whole coding region was identified by DNA sequencing (SEQ ID NO: 7). A fusion protein expressed from the fusion gene was designated "CTLA4-IgG", whose predicted amino acid sequence is represented by SEQ ID NO: 8.

EXAMPLE 2: Preparation of glycosylated CTLA4-IgG fusion proteins

In order to introduce a glycosylation motif into the Fc region of IgG1, seven primers having a nucleotide sequence containing a mutation leading to an amino acid substitution were prepared as follows: in the nucleotide sequence of SEQ ID NO: 5, a 478-480 codon (ATG, Met) was replaced with AAC (Asn, N), a 583-585 codon (GCC, Ala) with AAC (Asn, N), a 727-729 codon (ACC, Thr) with AAC (Asn, N), a 793-795 codon (GAG, Glu) with AAC (Asn, N), a 895-897 codon (TAC, Tyr) with ACC (Thr, T), a 991-993 codon (TTC, Phe) with ACC (Thr, T), and a 1036-1038 codon (CAG, Gln) with AAC (Asn, N). Information on these primers is given in Table 3, below.

TABLE 3

Information on primers used in the preparation of glycosylated CTLA4-Ig

Primer name	SEQ ID NO	Description
mg-hIgG1-CH2-1	9	Primer leading to an N (asparagine) substitution for M (methionine) at position 160 of SEQ ID NO. 6
mg-hIgG1-CH2-2	10	Primer leading to an N (asparagine) substitution for A (alanine) at position 195 of SEQ ID NO. 6
mg-hIgG1-CH2-3	11	Primer leading to an N (asparagine) substitution for T (threonine) at position 243 of SEQ ID NO. 6
mg-hIgG1-CH3-1	12	Primer leading to an N (asparagine) substitution for E (glutamic acid) at position 265 of SEQ ID NO. 6
mg-hIgG1-CH3-2	13	Primer leading to a T (threonine) substitution for Y (tryptophan) at position 299 of SEQ ID NO. 6
mg-hIgG1-CH3-3	14	Primer leading to a T (threonine) substitution for F (phenylalanine) at position 331 of SEQ ID NO. 6
mg-hIgG1-CH3-4	15	Primer leading to a N (aspargine) substitution for Q (glutamine) at position 346 of SEQ ID NO. 6

Glycosylated fusion proteins of the present invention were prepared by PCR using the cloning vector carrying CTLA4-hIgG-coding DNA, prepared in Example 1, as a template, and oligonucleotides listed in Table 3 as primers.

In detail, each glycosylated fusion protein was prepared as follows.

(1) CTLA4-hIgG-G1 (G1 variant): one glycosylation motif was created using a primer (SEQ ID NO: 9) designed to have a nucleotide sequence containing a substitution of AAC (Asn, N) for 478-480 nucleotides (ATG, Met) positioned at the Fc region of IgG (SEQ ID NO: 5).

(2) CTLA4-hIgG-G2 (G2 variant): two glycosylation motifs were created using primers (SEQ ID NOS: 12 and 13) designed to have nucleotide sequences containing substitutions of AAC (Asn, N) and ACC (Thr, T) for 793-795 nucleotides (GAG, Glu) and 895-897 nucleotides (TAC, Tyr), respectively, positioned at the Fc region of IgG (SEQ ID NO: 5).

(3) CTLA4-hIgG-G3 (G3 variant): two glycosylation motifs were created using primers (SEQ ID NOS: 13 and 14) designed to have nucleotide sequences containing substitutions of ACC (Thr, T) and ACC (Thr, T) for 895-897 nucleotides (TAC, Tyr) and 991-993 nucleotides (TTC, Phe), respectively, positioned at the Fc region of IgG (SEQ ID NO: 5).

(4) CTLA4-hIgG-G4 (G4 variant): three glycosylation motifs were created using primers (SEQ ID NOS: 9, 12 and 13) designed to have nucleotide sequences containing substitutions of AAC (Asn, N), AAC (Asn, N) and ACC (Thr, T) for 478-480 nucleotides (ATG, Met), 793-795 nucleotides (GAG, Glu) and 895-897 nucleotides (TAC, Tyr), respectively, positioned at the Fc region of IgG (SEQ ID NO: 5).

(5) CTLA4-hIgG-G5 (G5 variant): four glycosylation motifs were created using primers (SEQ ID NOS: 9, 12, 13 and 14) designed to have nucleotide sequences containing substitutions of AAC (Asn, N), AAC (Asn, N), ACC (Thr, T) and ACC (Thr, T) for 478-480 nucleotides (ATG, Met), 793-795 nucleotides (GAG, Glu), 895-897 nucleotides (TAC, Tyr) and 991-993 nucleotides (TTC, Phe), respectively, positioned at the Fc region of IgG (SEQ ID NO: 5).

(6) CTLA4-hIgG-G6 (G6 variant): five glycosylation motifs were created using primers (SEQ ID NOS: 9, 10, 12, 13 and 14) designed to have nucleotide sequences containing substitutions of AAC (Asn, N), AAC (Asn, N), AAC (Asn, N), ACC (Thr, T) and ACC (Thr, T) for 478-480 nucleotides (ATG, Met), 583-585 nucleotides (GCC, Ala), 793-795 nucleotides (GAG, Glu), 895-897 nucleotides (TAC, Tyr) and 991-993 nucleotides (TTC, Phe), respectively, positioned at the Fc region of IgG (SEQ ID NO: 5).

(7) CTLA4-hIgG-G7 (G7 variant): six glycosylation motifs were created using primers (SEQ ID NOS: 9, 10, 11, 12, 13 and 14) designed to have nucleotide sequences containing substitutions of AAC (Asn, N), AAC (Asn, N), AAC (Asn, N), AAC (Asn, N), ACC (Thr, T) and ACC (Thr, T) for 478-480 nucleotides (ATG, Met), 583-585 nucleotides (GCC, Ala), 727-729 nucleotides (ACC, Thr),

793-795 nucleotides (GAG, Glu), 895-897 nucleotides (TAC, Tyr) and 991-993 nucleotides (TTC, Phe), respectively, positioned at the Fc region of IgG (SEQ ID NO: 5).

(8) CTLA4-hIgG-G8 (G8 variant): seven glycosylation motifs were created using primers (SEQ ID NOS: 9, 10, 11, 12, 13, 14 and 15) designed to have nucleotide sequences containing substitutions of AAC (Asn, N), AAC (Asn, N), AAC (Asn, N), AAC (Asn, N), ACC (Thr, T), ACC (Thr, T) and AAC (Asn, N) for 478-480 nucleotides (ATG, Met), 583-585 nucleotides (GCC, Ala), 727-729 nucleotides (ACC, Thr), 793-795 nucleotides (GAG, Glu), 895-897 nucleotides (TAC, Tyr), 991-993 nucleotides (TTC, Phe) and 1036-1038 nucleotides (CAG, Gln), respectively, positioned at the Fc region of IgG (SEQ ID NO: 5).

The PCR was carried out as follows. To a PCR tube, 1 μl of CTLA4-hIgG DNA (2.2ng), 1.25 U Pfu DNA polymerase (Stratagene, USA), 4U Pfu DNA ligase (Stratagene, USA), 1 μl of 10× reaction buffer for Pfu DNA ligase, 1 μl of each primer (10 pM), and 2 μl of dNTP (each 10 mM) were added, and triple distilled water was added to a final volume of 20 μl. PCR conditions included two cycles of 3 min at 94°C, 1 min at 61°C and 1 min at 65°C, and then 29 cycles of 1 min at 94°C, 1 min at 61°C and 7 min at 65°C, followed by final elongation at 65°C for 15 min. The PCR products thus obtained were subjected to sequence analysis to determine whether a glycosylation motif was successfully inserted.

EXAMPLE 3

A. Expression and purification of glycosylated CTLA4-IgG fusion proteins

To express glycosylated CTLA4-IgG fusion proteins in Chinese hamster ovary K-1 cells (CHO-K1, ATCC CCL-61, Ovary, Chinese hamster *Cricetulus griseus*), pBluescript KS II(+) plasmid DNA containing a CTLA4-hIgG fusion gene into which a glycosylation motif was inserted was isolated from transformed *E. coli*, and digested with EcoRI and XbaI. The thus-obtained CTLA4-

hIgG fusion gene was inserted into EcoRI/XbaI sites of an animal expression vector, pCR™ 3 (Invitrogen, USA). The resulting expression vectors were designated as pCT4Ig-G2 to G8 plasmids. Among them, the pCT4Ig-G2 recombinant expression vector was deposited at the Korean Culture Center of Microorganisms (KCCM) on May 17, 2004 under the provisions of the Budapest Treaty and assigned accession number KCCM 10572.

B. Transfection and evaluation of expression of fusion genes

Chinese hamster ovary K-1 cells (CHO-K1) were plated onto six-well tissue culture plates (Nunc, USA) at a density of 1-3×10^5 cells per well, and were grown to a 50-80% confluency in 10% FBS-containing DMEM medium. In a serum-free DMEM, 1-2 μg DNA of any one of pCT4Ig-G2 to G8 plasmids was mixed with 2-25 μl of lipofectamine (Gibco BRL, USA), and incubated at room temperature for 15-45 min to form DNA-liposome complexes. Then, the resulting complex was added to the six-well plates. After an incubation period of 5 hrs, the cells were refed with 20% FBS-containing DMEM medium and further cultured for 18-24 hrs. Thereafter, the cells were cultured in 10% FBS-containing DMEM supplemented with 3 mg/ml geneticin (G418, Gibco BRL, USA) for three weeks. Formed colonies were selected and isolated, and then propagated.

Whether or not a fusion gene was expressed was evaluated by ELISA using peroxidase-labeled goat anti-human IgG (KPL, USA). ELISA was carried out as follows. First, 1 mg/ml of goat anti-human IgG was diluted to 1:2000 with 0.1 M sodium bicarbonate, and 100 μl of the diluent was aliquotted into a 96-well flexible plate (Falcon, USA). After being sealed with saran wrap, the plate was incubated at 4°C for over 16 hrs to allow the bottom of the plate to be coated with the antibody. Then, the plate was washed three times with a washing buffer (1× 0.1% Tween-20-containing phosphate buffered saline (PBS)), and 100 μl of a dilution buffer (48.5 ml 1× PBS, 1.5 ml FBS, 50 μl Tween-20) was added to each well. 20 μl of a culture supernatant was added to the first well and serially diluted using a micropipette. 0.01 μg/μl of human IgG (Sigma, USA) as a positive

control and a culture fluid of non-transfected CHO-K1 cells as a negative control were also diluted like the test sample. After dilutions were completed, the 96-well flexible plate (Falcon, USA) was wrapped with foil, incubated at 37°C for 1 hr 30 min and washed with the washing buffer three times. Peroxidase-labeled goat anti-human IgG (KPL, USA) was diluted to 1:5000 with the diluent buffer, and 100 μl of the diluent was added to each well, wrapped with foil and incubated at 37°C for one hour. After the reaction was completed, the plate was developed with a TMB microwell peroxidase substrate system (KPL, USA). Absorbance was measured at 630 nm using a microplate reader (Bio-RAD, Model 550, Japan) to determine whether a fusion gene was expressed and the expression levels of the fusion gene (FIG. 2).

As shown in FIG. 2, the G1 variant was expressed in the highest levels, followed by G2, G4, G0 and G3 variants. The G5, G6, G7 and G8 variants were found to be rarely expressed.

EXAMPLE 4

A. Western blot analysis

An expressed protein was purified by immunoprecipitation and subjected to Western blotting. First, 50 μl of protein A-Sepharose beads were placed into a 1.5-ml tube, mixed with 100 μl of buffer A (0.05 M boric acid, 4 M NaCl, pH 9.0), and centrifuged at 13,000 rpm for about 10 sec. After the supernatant was discarded, this step was repeated three times. Each protein sample was mixed with the equilibrated protein A-Sepharose beads and incubated at 4°C for 3 hrs with rotation to induce binding. Then, the reaction mixture was centrifuged at 13,000 rpm, and the beads were washed with buffer A three times. The beads were mixed with 20 μl of buffer B (0.05 M sodium phosphate, 0.05 M citric acid, 0.3 M NaCl, pH 3.0), and centrifuged at 13,000 rpm to elute bound proteins. The eluted protein sample was mixed with 5× buffer containing 5% β-mercaptoethanol, boiled for 5 min, and subjected to reduced SDS-PAGE. A 3.5% Acrylamide gel (0.5 M Tris-HCl (pH 6.8), 0.4% SDS)

was used as a stacking gel, and a 10% Acrylamide gel (1.5 M Tris-HCl (pH 8.8), 0.4% SDS) was used as a running gel. After electrophoresis, proteins were electro-transferred onto a 0.4-μm Westran (PVDF transfer membrane, S&S) for 2 hrs at 350 mA. The blot was blocked with 5% skim milk for 1 hr. After being washed with washing buffer (0.1% Tween-20, 1× phosphate buffered saline) three times, the blot was incubated in a 1:2000 dilution of peroxidase-labeled goat anti-human IgG (KPL, USA) for 1 hr. The blot was washed with washing buffer three times, and developed at room temperature for 10 min with 15 ml of a coloring agent, which was made according to a recommended usage method using a DAB substrate kit (VECTOR LABORATORIES, USA). The reaction was terminated with triple-distilled water. The results are given in FIG. 3.

EXAMPLE 5: Measurement of serum half-lives of glycosylated CTLA4-hIgG fusion proteins in mice

Serum half-lives of glycosylated CTLA4-hIgG fusion proteins were measured in mice as follows. Each fusion protein was intraperitoneally injected into mice (ICR, Samtako Inc., Korea) in a dose of 0.2 mg/kg. Blood samples were collected at given points in time for a minimum of 50 hrs, and protein concentrations were determined according to the same ELISA procedure as in Example 3 (FIG. 4).

As shown in FIG. 4, the G2, G3 and G4 variants had increased serum levels, whereas the G1 variant displayed reduced blood circulation time compared to the wild type. In particular, the G2 variant exhibited the highest circulation time.

Industrial Applicability

As described hereinbefore, the glycosylated fusion proteins according to the present invention are able to reduce dosage and administration frequency in clinical applications because they have high *in vivo* stability.

Claims

1. A glycosylated immunoglobulin or a fragment thereof, in which an immunoglobulin variant, comprising one or more amino acid modifications selected from the group consisting of M160N, A195N, T243N, E265N, Y299T, F331T and Q346N, is additionally glycosylated.

2. The glycosylated immunoglobulin or the fragment thereof according to claim 1, wherein the immunoglobulin variant comprises an amino acid sequence of SEQ ID NO: 19.

3. The glycosylated immunoglobulin or the fragment thereof according to claim 1 or 2, wherein the fragment is an Fc portion.

4. A DNA encoding a glycosylated immunoglobulin or a fragment thereof, in which an immunoglobulin variant, comprising one or more amino acid modifications selected from the group consisting of M160N, A195N, T243N, E265N, Y299T, F331T and Q346N, is additionally glycosylated.

5. The DNA according to claim 4, wherein the immunoglobulin variant comprises an amino acid sequence of SEQ ID NO: 19.

6. The DNA according to claim 4 or 5, wherein the fragment is an Fc portion.

7. A glycosylated fusion protein formed as a result of linkage of (a) a glycosylated immunoglobulin or a fragment thereof, in which an immunoglobulin variant having a modified amino

acid sequence forming one or more Asn-X-Ser/Thr sequences (X is any amino acid excluding proline) is additionally glycosylated, with (b) at least one biologically active protein or a portion thereof.

8. The glycosylated fusion protein according to claim 7, wherein the immunoglobulin variant comprises one or more amino acid modifications selected from the group consisting of M160N, A195N, T243N, E265N, Y299T, F331T and Q346N.

9. The glycosylated fusion protein according to claim 7, wherein the immunoglobulin variant comprises an amino acid sequence of SEQ ID NO: 19.

10. The glycosylated fusion protein according to claim 7, wherein the fragment is an Fc portion.

11. The glycosylated fusion protein according to claim 7, wherein the portion of the biologically active protein is a soluble extracellular domain.

12. The glycosylated fusion protein according to claim 7 or 11, wherein the biologically active protein is selected from the group consisting of hemoglobin, serum proteins, cytokines, α-, β- and γ-interferons, colony-stimulating factors, platelet-derived growth factor (PDGF), phospholipase activating proteins (s), insulin, plant proteins, tumor necrosis factor (TNF) and its mutant alleles, growth factors, hormones, calcitonin, calcitonin gene related peptide (CGRP), synthetic enkephalin, somatomedin, erythropoietin, hypothalamus releasing factors, prolactin, chronic gonadotrophin, tissue plasminogen-activating agents, growth hormone-releasing peptide (GHRP), thymic humoral factor (THF), interleukins, interferons, and enzymes.

13. A glycosylated fusion protein in a dimeric form in which two molecules of the glycosylated fusion protein according to any one of claims 7 to 12 are linked by a disulfide bond at a hinge region.

14. A DNA encoding the glycosylated fusion protein according to any one of claims 7 to 12.

15. A recombinant expression vector comprising the DNA encoding the fusion protein according to claim 14.

16. The recombinant expression vector according to claim 15, which has accession number KCCM-10572.

17. A host cell transformed or transfected with the recombinant expression vector according to claim 15 or 16.

18. A method of preparing a glycosylated fusion protein, comprising culturing the transformed or transfected host cell according to claim 17 and isolating the glycosylated fusion protein from a resulting culture.

19. A pharmaceutical composition comprising the glycosylated fusion protein according to any one of claims 7 to 13.