Novel Natural Protein and Application thereof

BACKGROUND

The present application relates generally to a novel protein and application thereof, the secretory protein can be used to prepare the drugs for preventing and treating the diseases induced by excessive formation or excessive accumulation of junk proteins.

DESCRIPTION OF RELATED ART

In normal physiological activities, the organism generates lots of junk proteins, including oxidation protein, glycosylated protein and some abnormal spliced proteins (polypeptide). The organism retains multiple mechanisms for removing junk proteins to maintain normal physiological function. However, the aging or diseases will induce excessive formation of junk proteins or degrade the organism's ability to remove junk proteins, so that lots of junk proteins accumulate. The abnormal accumulation of junk proteins inside or outside the cells is the key mechanism inducing a series of diseases. The typical diseases include chronic kidney disease, Alzheimer's disease (AD), Huntington's disease, diabetes complications and so on [1-5]. The accumulation of oxidation protein, glycated protein or other junk proteins in the circulatory system is one of the key causes for the aging of organism [6-7]. It is proved by research that the advanced oxidation protein (AOPP) in the serum damages renal cells, and it is the main pathogenesis of chronic kidney disease. The AOPP in serum can induce the programmed apoptosis of islet β cells [3, 8]. The β amyloid hypothesis indicates that the synaptic dysfunction and neuron death resulted from progressive accumulation of toxoprotein induced by unbalance of generation and removal of Aβ protein in tissues are the first causes of AD [9]. The amylin protein not only performs

abnormal aggregation in the insular tissues of partial diabetics, but also exists in the plaques of brain tissue, and it is closely related to the progress of diabetes and neurodegenerative diseases [10, 11]. Based on these findings, in some disease models, the Aβ aggregation or formation in the AD animal pattern is blocked by using antibody [12], polypeptide drug [13] or micromolecular drug [14], the formation of nervous tissue plaques can be reduced, and the animal cognition level is increased. These results show that using drugs to depress the formation and aggregation of these pathogenic proteins or to promote the removal of pathogenic proteins to reduce the accumulation of pathogenic proteins is an important method to prevent or treat these diseases.

Previous research indicates that when the oxidative stress occurs in the cell, the DSS1 (deleted split hand/split foot 1) protein, as a highly conservative small protein in eukaryote, can be covalently modified to oxidation protein under the conditions of enzymatic reaction and ATP consumption, such a modification will mediate the oxidation protein to degrade in the cell [15]. The DSS1 gene knockout leads to cell death; the cells with high expression of DSS1 protein manifest significant resistance to the oxidative stress or antineoplastics-induced cell apoptosis [16]. These results show the vital function of DSS1 protein in the course of removing oxidation protein from cells, and it is the key to the existence of cells.

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SUMMARY OF THE APPLICATION

In the latest study, we (inventors) have found that there is a new subtype of DSS1 protein in higher primate (anthropoid subfamily) genome, named secretory DSS1 protein (sDSS1). The sDSS1 is the first DSS1 protein subtype discovered, and its sequence, properties and function are highly similar to DSS1. However, it can be secreted into blood and cerebral spinal fluid, its properties are more active, and can form a polymer with the oxidation protein in serum or buffer solution without energy-consuming enzymatic reaction, or combine with Aβ protein and depress the formation of Aβ oligomer. The sDSS1 protein added to the culture medium can shield the cytotoxicity induced by oxidation protein, Aβ oligomer, amylin oligomer and glycosylated protein to protect cell viability. Therefore, we identify this new type of protein sDSS1 as a promising drug for preventing and

treating the diseases induced by oxidation protein, glycosylated protein, Aβ, amylin and other pathogenic proteins with similar features.

In one aspect, provided herein is a secretory deleted split hand/split foot 1 (sDSS1) protein for use in therapy.

In another aspect, provided herein is a use of a secretory deleted split hand/split foot 1 (sDSS1) protein in the manufacture of a medicament for reducing apoptosis or promoting cell survival of a live cell that is exposed to a pathogenic polypeptide.

The specific technical solution is described below:

A novel sDSS1 protein is provided, which includes the natural protein sDSS1 present in Anthropoidea animals including humans, wherein a human sDSS1 protein has an amino acid as set forth in SEQ ID NO: 1.

Preferably, the Anthropoidea animals may further be chimpanzee, bonobo, gorilla, orangutan, white-cheeked gibbon, golden snub-nosed monkey, rhesus macaque, olive baboon, Angola colobus, sooty mangabey, drill and northern pigtail macaque; wherein a chimpanzee sDSS1 protein has an amino acid as set forth in SEQ ID NO: 5, a bonobo sDSS1 protein has an amino acid as set forth in SEQ ID NO: 6, a gorilla sDSS1 protein has an amino acid as set forth in SEQ ID NO: 7, an orangutan sDSS1 protein has an amino acid as set forth in SEQ ID NO: 8, a white-cheeked gibbon sDSS1 protein has an amino acid as set forth in SEQ ID NO: 9, a golden snub-nosed monkey sDSS1 protein has an amino acid as set forth in SEQ ID NO: 10, a rhesus macaque sDSS1 protein has an amino acid as set forth in SEQ ID NO: 11, an olive baboon sDSS1 protein has an amino acid as set forth in SEQ ID NO: 12, a Angola colobus sDSS1 protein has an amino acid as set forth in SEQ ID NO: 13, a sooty mangabey sDSS1 protein has an amino acid as set forth in SEQ ID NO: 14, a drill sDSS1 protein has an amino acid as set forth in SEQ ID NO: 15, a northern pigtail macaque sDSS1 protein has an amino acid as set forth in SEQ ID NO: 16.

Preferably, the sDSS1 protein includes a N-terminal amino acid sequence of 58 amino acids and a C-terminal amino acid sequence of 31 amino acids, wherein the human sDSS1 protein has a N-terminal amino acid sequence of 58 amino acids as set forth in SEQ ID NO: 3, the human sDSS1 protein has a C-terminal amino acid sequence of 31 amino acids as set forth in SEQ ID NO: 2; wherein the N-terminal amino acid sequence of the 58 amino acids includes 3 or more amino

acid sequences with consecutive acidic amino acids, each of amino acid sequences with consecutive acidic amino acids includes no more than 10 acidic amino acids, any two adjacent amino acid sequences of the amino acid sequences with consecutive acidic amino acids have a spacing of no more than 4 amino acids, and the spacing includes at least one hydrophobic amino acid, a pH value is not higher than 4.5, the N-terminal amino acid sequence of the 58 amino acids includes no less than 10 acidic amino acids; the C-terminal amino acid sequence following position 58 of the N-terminal amino acid sequence of the 58 amino acids are relatively hydrophobic overall, the C-terminal amino acid sequence of the 31 amino acids includes no less than 10 hydrophobic amino acids;

wherein the hydrophobic amino acid is selected from the group consisting of alanine, isoleucine, leucine, valine, cysteine, phenylalanine, methionine, tryptophan, and tyrosine;

the neutral amino acid is selected from the group consisting of threonine, glycine, serine, histidine, and glutamine;

the acidic amino acid is selected from the group consisting of glutamic acid, aspartate, proline, and asparaginate; and

the basic amino acids is selected from the group consisting of arginine, and lysine.

Preferably, the sDSS1 protein in the Anthropoidea animals includes a C-terminal amino acid sequence of:

$X_1X_2X_3X_4X_5X_6X_7X_8X_9X_{10}X_{11}X_{12}X_{13}X_{14}X_{15}X_{16}X_{17}X_{18}X_{19}X_{20}X_{21}X_{22}X_{23}X_{24}X_{25}X_{26}X_{27}X_{28}X_{29}X_{30}X_{31}$;

X_1 is a neutral amino acid; X_2 is a hydrophobic amino acid; X_3 and X_4 are hydrophobic amino acids; X_5 is a hydrophobic amino acid; X_6 is a hydrophobic amino acid; X_7 is a hydrophobic amino acid; X_8 is a hydrophobic amino acid; X_9 is a hydrophobic amino acid; X_{10} is an acidic amino acid; X_{11} is a neutral amino acid; X_{12} is a hydrophobic amino acid; X_{13} is a hydrophobic amino acid; X_{14} is a neutral

amino acid; X_{15} is a hydrophobic amino acid; X_{16} is a hydrophobic amino acid; X_{17} is a hydrophobic amino acid; X_{18} is a hydrophobic amino acid; X_{19} is a basic amino acid; X_{20} is an acidic amino acid; X_{21} is a basic amino acid; X_{22} is a neutral amino acid; X_{23} is a basic amino acid; X_{24} is a hydrophobic amino acid; X_{25} is a hydrophobic amino acid; X_{26} is a neutral amino acid; X_{27} is a hydrophobic amino acid; X_{28} is a hydrophobic amino acid; X_{29} is a hydrophobic amino acid; X_{30} is a hydrophobic amino acid; and X_{31} is a hydrophobic amino acid;

an amino acid sequence having 40% or more homology to the C-terminal amino acid sequence of the 31 amino acids, wherein the amino acid sequence has a same or similar property and function to a C-terminal amino acid sequence of a human sDSS1 protein.

A polypeptide encoded by a polypeptide sequence, wherein the polypeptide sequence is constructed based on the N-terminal amino acid sequence of the 58 amino acids and the C-terminal amino acid sequence of the 31 amino acids of the sDSS1 protein according to the above solution, wherein

1) the polypeptide sequence has a N-terminal having 40% or more similarity to the N-terminal amino acid sequence of the 58 amino acids, and the polypeptide sequence has a C-terminal having 40% or more similarity to the C-terminal amino acid sequence of the 31 amino acids, a protein encoded by the polypeptide sequence has a same or similar property and function to a human sDSS1 protein; or

2) a N-terminal of the polypeptide sequence is based on a N-terminal amino acid sequence of 58 amino acids of a human sDSS1 protein, or is a sequence having 40% or more similarity to the N-terminal amino acid sequence of the 58 amino acids of the human sDSS1 protein, wherein a C-terminal or the N-terminal of the polypeptide is fused with other amino acid sequence, the other amino acid sequence for fusion has an identical or similar property to a C-terminal amino acid sequence of 31 amino acids of the human sDSS1 protein and perform the same or

similar functions, a modified protein encoded by the polypeptide sequence performs an identical or similar function to the human sDSS1 protein; or

3) the peptide sequence is constructed by fusing the C-terminal amino acid sequence of the 31 amino acids in the sDSS1 protein according to the above solution with other polypeptide sequence.

The fusion protein includes a full sequence or a partial sequence of the sDSS1 protein according to the above solution, and the polypeptide sequence according to the above solution.

Preferably, the fusion protein is a protein complex formed by linking the protein sDSS1 protein, a carrier protein, an antibody or other arbitrary amino acid sequence.

A complex includes a full sequence or a partial sequence of the sDSS1 protein according to the above solution, the polypeptide sequence of the above solution, or a full sequence or a partial sequence of the fusion protein according to the above solution.

Preferably, the complex is a complex formed by linking the sDSS1 protein to a pharmaceutically acceptable drug carrier.

Preferably, the pharmaceutically acceptable drug carrier includes one or more of a microsphere/capsule, liposome, micro-emulsion, nanoparticle, magnetic particle and gel.

A nucleotide encodes the sDSS1 protein according to the above solution, or the polypeptide according to the above solution.

Preferably, the nucleotide includes DNA and RNA.

A cell expresses the sDSS1 protein according to the above solution or the polypeptide according to the above solution.

Preferably, the cell is a stem cell, a precursor cell or an adult cell of a mammal.

Preferably, the mammal is a human, an orangutan, a monkey, a horse, a cattle, a sheep, a pig, a donkey, a dog, a rabbit, a cat, a rat or a mouse.

Preferably, the cell includes an embryo stem cell, an induced multipotential stem cell or a stem cell derived from a primary culture, a multipotential or monopotential stem cell derived from a mother cell differentiation.

An expression system, wherein a nucleotide sequence coding the sDSS1 protein according to the above solution or the polypeptide according to the above solution is introduced into an organism, and the sDSS1 protein according to the above solution or the polypeptide according to the above solution is expressed in the organism.

Preferably, the expression system is selected from the group consisting of eukaryotic expression plasmid vector, adenovirus, slow virus, retrovirus, CRISPR/Cas technique and other feasible gene-editing techniques.

Preferably, the organism is a human, an orangutan, a monkey, a horse, a cattle, a sheep, a pig, a donkey, a dog, a rabbit, a cat, a rat, a mouse, a chicken, a duck or a goose.

A drug primarily targets the sDSS1 protein according to the above solution or the polypeptide according to the above solution, wherein the drug can affect an expression level of the sDSS1 protein according to the above solution or the polypeptide according to the above solution in the organism upon administration.

Preferably, the drug is a chemical micromolecular drug, a protein/polypeptide drug, a nucleic acid drug, or a nanodrug.

Preferably, the nucleic acid drug includes one or more of a siRNA, a microRNA, an antisense oligonucleotide, a triple strand DNA and a ribozyme.

A method of producing a protein, includes the following steps:

S1. constructing an expression vector: inserting a nucleotide sequence coding the sDSS1 protein according to the above solution or the polypeptide according to

the above solution into a plasmid and introducing the plasmid into bacteria or yeast cell, or inserting the nucleotide sequence coding the sDSS1 protein according to the above solution or the polypeptide according to the above solution into genome of an insect cell or a mammalian cell;

S2. expressing the sDSS1 protein: expanding a culture of the bacteria, yeast cell, insect cell or mammalian cell as modified in S1, and collecting a culture medium or cell lysate containing the sDSS1 protein according to the above solution or the polypeptide according to the above solution;

S3. purifying the sDSS1 protein: coarse filtering and purifying the culture medium or cell lysate obtained in S2 to obtain the sDSS1 protein.

A method of producing a protein, includes using chemical synthesis technique to produce the sDSS1 protein according to the above solution or the polypeptide according to the above solution.

A method of producing a protein, includes using *in vitro* ribosome expression system to produce the sDSS1 protein according to the above solution or the polypeptide according to the above solution.

An application of the sDSS1 protein, polypeptide, fusion protein, complex, nucleotide sequence, cell, expression system, or drug according to the above solution in diagnosing, preventing or treating disease.

Preferably, the disease is a disease induced by excessive formation or accumulation of pathogenic protein/polypeptide.

Preferably, the pathogenic protein/polypeptide is an oxidation protein product, glycosylation protein product, an amyloid precursor protein and a spliceosome thereof, an islet amyloid polypeptide and a spliceosome thereof, or other pathogenic protein/polypeptides having features similar to an oxidation protein a glycosylation protein, an amyloid protein or an islet amyloid polypeptide.

Preferably, the diagnosing of the disease includes detecting one or more of an expression level of a full or partial sequence of the amino acid sequence, mRNA level and number of gene copies of the sDSS1 protein according to the above solution.

Preferably, the preventing includes one or more of genetic modification, nucleic acid introduction, drug injection/administration, cellular transplantation and tissue transplantation.

Preferably, the treating includes one or more of genetic modification, nucleic acid introduction, drug injection/administration, cellular transplantation and tissue transplantation.

The characteristics and/or beneficial effects of the present application are:

1. The polypeptide sequence of the sDSS1 protein and typical human sDSS1 protein provided by the present application is MSEKKQPVDLGLLEEDDEFEEFPAEDWAGLDEDEDAHVWEDNWDDDNV EDDFSNQLRATVLLMILVCETPYGCYVLHQKGRMCSAFLCC (see SEQ ID NO: 1). According to bioinformatic analysis, the protein is a protein of anthropoid subfamily animals.

2. According to bioinformatic analysis and cell experiment, the 31-amino acid carbon terminal sequence of the sDSS1 protein is a signal peptide and has critical effect on the properties and secretion property of the protein. The C-terminal sequence of the 31 amino acids is TVLLMILVCETPYGCYVLHQKGRMCSAFLCC(see SEQ ID NO: 2).

3. The sDSS1 protein defined in the present application can be combined with oxidation protein, glycosylated protein, Aβ protein and amylin protein and shield the cytotoxicity induced by aggregation of these toxoproteins, so it has important potential in treating the diseases induced by excessive formation or excessive accumulation of these toxoproteins and other pathogenic proteins with similar features.

4. The sDSS1 protein of the present application is produced by fermentation of Escherichia coli. The nucleotide sequence coding the sDSS1 protein is inserted into pET151D plasmid, during sDSS1 expression, the nitrogen terminal is fused with a 6-his tag and a V5 tag for purification and immunoblotting detection. The expression of protein in Escherichia coli is preliminarily purified by using Ni-NTA gel column, and then the SDS-PAGE is used for gel purification. The cut strip containing His-V5-sDSS1 protein is put in a dialysis bag containing transfer buffer, the protein is extracted from the gel under the drive of electric field and collected in the dialysis bag. The protein purified by the SDS polyacrylamide gel electrophoresis analysis can reach the level for bioexperiment.

5. According to molecular experiment, the sDSS1 protein of the present application can be combined with the oxidation protein in serum and the oxidation protein in buffer solution to form polymers, or combine with Aβ protein to reduce the formation of Aβ oligomer.

6. The cell experiment proves that the sDSS1 protein of the present application can shield the cytotoxicity induced by oxidation protein, glycosylated protein, Aβ oligomer and amylin oligomer in the culture medium effectively, so as to maintain the cell viability.

To sum up, the present application provides a sDSS1 protein, the biological property and activity of sDSS1 protein are proved by the research in bioinformatics, molecular biology and cytobiology levels. The sDSS1 protein can reduce the cytotoxicity induced by oxidation protein, glycosylated protein, Aβ oligomer and amylin oligomer in culture medium effectively to maintain cell viability. As the sDSS1 protein is a congenital protein of higher primate, it is free of immunoreaction in clinical application. Therefore, the present application provides a candidate drug for preventing and treating the diseases induced by excessive formation or excessive accumulation of oxidation protein, glycosylated protein, Aβ protein, amylin polypeptide and other pathogenic proteins with similar features, and it has important application prospects in biomedicine.

BRIEF DESCRIPTION OF THE DRAWINGS

The present application is further explained by the following attached figures, so as to make the present application clear and complete, but not to limit the scope of protection of the present application.

Fig.1A. The sDSS1 gene is a new subtype of DSS1 gene, the comparison between human DSS1 gene cDNA (NM_006304.1, 509bp) and human sDSS1 gene cDNA (AK309241.1, 1195bp) shows an overlapping area, the nucleic acid sequence of the overlapping area can encode N-terminal 58 amino acid sequences according to analysis.

Fig.1B. The sDSS1 protein amino acid sequences of 13 species of primates were compared by using Clustal X2.1 software, the results show that the sDSS1 protein amino acid sequence is highly conservative, N-terminal 58 amino acid sequences are identical, and the C-terminal 31 amino acid sequences have point mutation only at a few sites.

Fig.2A. In plasmid transfected 293T cell, the GFP protein distribution was observed 24h later. The green fluorescence in control cell (GFP) was clear and bright, and the background in solution was dim. Obvious green fluorescence signal was observed in the culture solution of sDSS1 and GFP chelated protein (sDSS1-GFP) or sDSS1 protein C-terminal 31 amino acid sequences and GFP chelated protein (sDSS1-c-GFP), and the intracellular fluorescence disperses and the intensity declines, meaning that the GFP protein was taken out of the cell with the sDSS1 protein or sDSS1 protein C-terminal sequence secretion.

Fig.2B. Point membrane immunoblotting tests for detecting transfected cell culture medium, the results show that the GFP signal was detected in sDSS1-GFP and sDSS1-c-GFP culture media, and there was no obvious signal detected in blank control and GFP control group, proving that the sDSS1 protein is a secretory protein, and C-terminal 31 amino acid sequences are signal peptide.

Fig.3A. The sDSS1 signal can be detected in the human serum or human cerebral spinal fluid (CSF) sample by using specific antibody of sDSS1 protein C-terminal polypeptide sequence (antigen sequence: C-terminal 31 amino acid sequences of sDSS1 protein). The Human CSF sample was from senior citizens, the serum sample *a* was from the blood of a youth after strenuous exercise, the serum samples *b*, *c* and *d* were from the blood of youths in resting state.

Fig.3B. The specific mRNA sequence of sDSS1 gene (amplified product is 293bp) can be detected in human astrocytomas glioblastoma (U-87 MG) by using PCR, the DSS1 gene is used as control (amplified product is 238bp).

Fig.4A-Fig.4B. illustrates that coomassie brilliant blue staining shows the content of objective protein in the sDSS1 protein production and purification processes.

Fig.4A. The positive Escherichia coli cloning strain was selected to expand culture, the addition of IPTG can induce the expression of sDSS1 protein, the expression level of objective protein in the cell without induction was very low. **Fig.4B.** The concentrated lysate after preliminary purification of Ni-NTA gel column and the objective protein content after purification were tested, channel *a* shows the purified sDSS1 protein, channel *b* shows the preliminarily purified cell lysis solution.

Figs.5A-5D. illustrates that biochemical experiment and cell experiment prove that the sDSS1 protein can combine with oxidation protein and shield the toxicity of oxidation protein.

Fig.5A. The 0.72μg purified sDSS1 protein was mixed with different proportions of serum protein for incubation, the sDSS1 protein was tested by V5 conjugated protein (V5-HRP), the result shows that the sDSS1 protein and oxidation protein of serum formed macromolecular protein complex. **Fig.5B**. The AOPP (200 μg/mL) and the purified sDSS1 proteins at different concentrations were incubated at 4℃ over night, the product was separated by SDS-PAGE, and

the Coomassie brilliant blue staining shows that the sDSS1 protein and AOPP can form macromolecular complex, the complex content increases with sDSS1 protein concentration. **Fig.5C**. The culture medium is mixed with 10% oxidized serum, the cell proliferation was reduced significantly, the sDSS1 protein in culture medium can shield the cytotoxicity derived from the oxidized serum. **Fig.5D**. The culture medium without serum was mixed with 100μg/mL AOPP protein to reduce the cell viability, the addition of sDSS1 protein at isoconcentration can retrieve cell viability, the 100μg/mL BSA was used for control group. The data was analyzed by t-test two-tailed test and validated by ANOVE. **, p-value < 0.01.

Figs.6A-6E. illustrates that the sDSS1 protein reduces the formation of Aβ oligomer, and reduces the cytotoxicity and cell apoptosis induced by Aβ oligomer.

Fig.6A. Different proportions of sDSS1 protein were mixed with 10μg Aβ protein before incubation, according to Aβ antibody test, the sDSS1 protein and Aβ formed covalently conjugated high molecular weight protein complex, such a conjugation can reduce the formation of Aβ oligomer with cytotoxicity. **Fig.6B**.V5 sDSS1 protein was tested by conjugated protein (V5-HRP), the result shows that the sDSS1 protein and Aβ formed a protein complex. **Fig.6C**. The addition of Aβ oligomer to the culture medium induced cytotoxicity, the cell viability was degraded, the sDSS1 protein can shield the cytotoxicity induced by Aβ oligomer completely. **Fig.6D**. The cell apoptosis experiment shows that the sDSS1 protein added to the culture medium reduced the early apoptosis and late apoptosis of SH-SY5Y cells induced by Aβ oligomer significantly, so as to reduce the effect of toxoprotein on cells. **Fig.6E**. The sDSS1 protein can shield the toxicity of Aβ oligomer for mouse nerve stem cells (NSCs). The data was analyzed by t-test two-tailed test and validated by ANOVE. **, p-value < 0.01.

Fig.7. The amylin oligomer added to the culture medium induces cytotoxicity and reduces cell viability, the addition of sDSS1 protein can retrieve the cell viability, promoting the cell survival. The data was analyzed by t-test two-tailed test and validated by ANOVE. **, p-value < 0.01.

Fig.8. The cell viability decline induced by 400 μg/mL glycosylated protein can be retrieved by sDSS1 protein, the retrieving effect increased with the sDSS1 protein concentration (100μg/mL to 200μg/mL). The 400μg/mL BSA protein was used for control group. The data was analyzed by t-test two-tailed test and validated by ANOVE. **, p-value < 0.01.

Fig.9A. illustrates that Operating method of injecting virus into the lateral ventricle of SAMP8 mouse and virus injection site.

Fig.9B. The adenovirus was injected into the lateral ventricle of a 5 months old senescence-accelerated mouse SAMP8 mouse (1μL virus into right and left brains respectively), the animal survival was observed continuously. The result shows that the survival rate after operation of the mouse injected with adenovirus expressing sDSS1 protein was apparently higher than that of control mouse (expressing GFP protein). The data was analyzed by ANOVE. **, p-value < 0.01.

DETAILED DESCRIPTION OF THE EMBODIMENTS

The preferred solutions of the present application are described and validated with examples in the following text, not to limit the scope of the present application. All scope of the present application are subject to the scope of the Claims.

The experimental methods for the following cases are conventional experimental methods unless otherwise specified.

In the following embodiments, the sDSS1 protein was produced in-house and its purity reached the level for bioexperiment, the other materials and reagents were commercially available.

Example 1, sDSS1 protein is a secretory protein of primate

Bioinformatic analysis tool:

National Center of Biotechnology Information (NCBI) genome database; Nucleotide blast tool (NCBI); Align sequences nucleotide blast tool (NCBI), Translate tool (SIB Bioinformatics Resource Portal); Clustal X2.1: Multiple Sequence Alignment (EMBL-EBI); SecretomeP 2.0 (CBS prediction service); WoLF PSORT II.

Bioexperimental method:

1. Cell culture, the 293T cells were bought from American type culture collection (ATCC), the cells were cultured in the cell culture medium containing 90% basal medium (Dulbecco's modified eagle medium, DMEM) (Life technology C#12500062) and 10% Fetal bovine serum (FBS) (Gibco C#10100-147), cultured in cell incubator (temperature 37℃, humidity 95%, CO2 concentration 5%), subcultured once every two days.

2. Cell transfection, the 293T cells were inoculated in a 6-well plate as per $3x10^5$ per well, mixed with 1.5mL cell culture medium, the plasmid was transfected when the cells have been adhering to the wall for 12 hours. The eukaryotic expression plasmid pCMV-C-Flag was used, the inserted nucleic acid sequence expressing sDSS1 protein was expressed as SEQ ID NO: 17. 2500ng of the plasmid was diluted and mixed with 750μL Opti-MEM®Medium (Life technology C#31985062) uniformly, 10μL transfection reagent Lip2000 (Invitrogen C#12566014) was diluted and mixed with 750μL Opti-MEM®Medium uniformly, the diluted plasmid solution was instilled into the diluted transfection reagent drop by drop, mixed uniformly and incubated at normal temperature for 5 minutes. The cell culture medium was blotted from the 6-well plate, the cells were cleaned with PBS, and then the incubated transfection working fluid was applied. The cells were cultured in the incubator continuously, the fluorescent protein expression of the cells 2was observed during 24 to 48 hours.

3. Western blotting, the PVDF membrane was activated by methanol and dried, the control culture medium and different transfection cell culture media were dripped onto the membrane. When the membrane was dried, the PVDF membrane completed 1%BSA sealing, primary antibody (Rabbit-anti-GFP) (Cell signal technology C#2956) incubation, secondary antibody (Goat-anti-rabbit HRP antibody) (Zsbio, ZDR-5403) incubation in turn. The membrane was cleaned with PBST three times, developed by luminescent liquid (Zsbio, ZLI-9017) and the bands were exposed by X-ray film.

Result analysis:

In the bioinformatic analysis of shfm1 gene in human genome, it was found that the gene has multiple transcripts (see shfm1 gene information in NCBI database, http://www.ncbi.nlm.nih.gov/gene/7979). Besides an mRNA sequence of jointly coded DSS1 protein sequence (NM_006304.1, 509bp), there is a longer mRNA sequence (AK309241.1, 1195bp). The short mRNA sequence and long mRNA sequence only have 256bp repeat sequence (Fig. 1A). According to nucleic acid sequence analysis, it can be seen from the Translate tool that the repeat sequence can encode DSS1 protein N-terminal 58 amino acid sequences. The long mRNA sequence coded for 89 amino acids. According to the alignment of polypeptide sequences, the long mRNA encoded polypeptide sequence and DSS1 polypeptide sequence have the overlapping area of N-terminal 58 amino acids, and the variation area of 31 amino acids. This new polypeptide was named secretory DSS1 protein (sDSS1), the polypeptide sequences are expressed as follows:

DSS1 (Homo sapiens):

MSEKKQPVDLGLLEEDDEFEEFPAEDWAGLDEDEDAHVWEDNWDD DNVEDDFSNQLRAELEKHGYKMETS (see SEQ ID NO: 4)

s-DSS1 (Homo sapiens):

MSEKKQPVDLGLLEEDDEFEEFPAEDWAGLDEDEDAHVWEDNWDD DNVEDDFSNQLRATVLLMILVCETPYGCYVLHQKGRMCSAFLCC (see SEQ ID NO: 1)

According to the screening of the sequenced primate genome and other animal pattern genomes in NCBI database, only the genome of Anthropoidea animals has similar long mRNA sequence and polypeptide sequence similar to human sDSS1 protein, as shown in Table 1. The polypeptide sequence alignment results show that the sDSS1 protein sequence is highly conservative, the N-terminal 59 amino acid sequences are identical, the other C-terminal amino acid sequences have a little point mutation (Fig.1B).

Table 1

Primate	**Species**	**Amino acid sequence**
Haplorrhini	Homo sapiens	MSEKKQPVDLGLLEEDDEFEEFPAEDWAGLDEDEDAHVWEDNWDDDNVEDDFSNQLRATVLLMILVCETPYGCYVLHQKGRMCSAFLCC (see SEQ ID NO: 1)
	Pan troglodytes	MSEKKQPVDLGLLEEDDEFEEFPAEDWAGLDEDEDAHVWEDNWDDDNVEDDFSNQLRATVLLMILVCETPYGCYVLHQKGRMCSAFLCC (see SEQ ID NO: 5)
	Pan paniscus)	MSEKKQPVDLGLLEEDDEFEEFPAEDWAGLDEDEDAHVWEDNWDDDNVEDDFSNQLRATVLLMILVCETPYGCYVLHQKGRMCSAFLCC (see SEQ ID NO: 6)
	Gorilla gorilla)	MSEKKQPVDLGLLEEDDEFEEFPAEDWAGLDEDEDAHVWEDNWDDDNVEDDFSNQLRVTVLLMILVCETLYGCYVLHQKGRMCSAFLCC

	(see SEQ ID NO: 7)
Pongo abelii	MSEKKQPVDLGLLEEDDEFEEFPAEDWAGLDEDEDAHVWEDNW DDDNVEDDFSNQLRATILLMILVCETPYGCYVLHQKGRMCSAFLC C (see SEQ ID NO: 8)
Nomascus leucogenys	MSEKKQPVDLGLLEEDDEFEEFPAEDWAGLDEDEDAHVWEDNW DDDNVEDDFSNQLRATVLLMVLVCETPYGCYVLHQKERMCSAFL CC (see SEQ ID NO: 9)
Rhinopithecus roxellana	MSEKKQPVDLGLLEEDDEFEEFPAEDWAGLDEDEDAHVWEDNW DDDNVEDDFSNQLRATVLLMIKVYETPYGCYILHQKGRMCSAFL CC (see SEQ ID NO: 10)
Macaca mulatta	MSEKKQPVDLGLLEEDDEFEEFPAEDWAGLDEDEDAHVWEDNW DDDNVEDDFSNQLRATVLLMIKVYETPYGCYILHQKGRMCSAFL CC (see SEQ ID NO: 11)
Papio anubis	MSEKKQPVDLGLLEEDDEFEEFPAEDWAGLDEDEDAHVWEDNW DDDNVEDDFSNQLRATVLLMIKVYETPYGCYILHQKGRMCSAFL CC (see SEQ ID NO: 12)
Angola colobus	MSEKKQPVDLGLLEEDDEFEEFPAEDWAGLDEDEDAHVWEDNW DDDNVEDDFSNQLRATVLLMKKVYETPYGCYILHQKGRMCSAFL CC (see SEQ ID NO: 13)
sooty	MSEKKQPVDLGLLEEDDEFEEFPAEDWAGLDEDEDAHVWEDNW

	mangabey	DDDNVEDDFSNQLRATVLLMIKVYETPYGCYILHQKGRMCSAFLCC (see SEQ ID NO: 14)
	Mandrillus leucophaeus	MSEKKQPVDLGLLEEDDEFEEFPAEDWAGLDEDEDAHVWEDNWDDDNVEDDFSNQLRATVLLMIKVYETPYGCYILHQKGRMCSAFLCC (see SEQ ID NO: 15)
	Macaca nemestrina	MSEKKQPVDLGLLEEDDEFEEFPAEDWAGLDEDEDAHVWEDNWDDDNVEDDFSNQLRATVLLMIKVYETPYGCYILHQKGRMCSAFLCC (see SEQ ID NO: 16)

The sDSS1 protein amino acid sequence was analyzed by using two kinds of secretory protein analysis and prediction software, which are Wolf PSORT and SecretomeP 2.0. The prediction results show that the sDSS1 protein is located outside the cells, similar to multiple identified secretory proteins, it is estimated as a secretory protein (Table 2). According to the analysis result of Wolf PSORT software, the signal peptide cleavage site of sDSS1 protein is located between amino acids positions 58-59.

Table 2

Species Name	**SecretomeP 2.0** (Recommended threshold for secreted protein: 0.6)	**WoLF PSORT** (Numbers of similar secreted proteins)	**Predicted protein location**
Homo sapiens	0.85	28	Extracellular
Pan troglodytes	0.85	28	Extracellular
Pan paniscus	0.85	28	Extracellular
Nomascus leucogenys	0.85	27	Extracellular

Gorilla gorilla	0.752	23	Extracellular
Pongo abelii	0.86	27	Extracellular
Rhinopithecus roxellana	0.836	29	Extracellular
Macaca mulatta	0.836	29	Extracellular
Angola colobus	0.823	28	Extracellular
sooty mangabey	0.836	29	Extracellular
M. leucophaeus	0.836	29	Extracellular
Macaca nemestrina	0.836	29	Extracellular
Papio anubis	0.836	29	Extracellular

According to the bioinformatic analysis results, the complete sequence or C-terminal 31 amino acid sequences (31 amino acid sequences after amino acid position 58) of the protein are connected to green fluorescent protein (GFP) and expressed in 293T cells (sDSS1-GFP, sDSS1-c-GFP). The results show that the solution had green fluorescence, the background emitted light, and the fluorescence in the cells was dim. There was no fluorescence in the control group (GFP) solution, the background was very dark, the fluorescence in the cells was clear and bright (Fig. 2A). The cell culture medium was tested by point membrane immunoblotting, the GFP signal was detected in the cell culture media of sDSS1-GFP and sDSS1-c-GFP groups, but the signal was not detected in the control group (GFP) (Fig. 2B). To sum up these results, the sDSS1 protein is a sort of secretory protein, it can be synthesized in the cells and secreted out of the cells, the C-terminal 31 amino acid sequences of sDSS1 protein perform the function of signal peptide.

Example 2, sDSS1 protein is a sort of protein

1. Human serum and CSF sample treatment. Fresh human whole blood was collected, kept still at room temperature for 10-20 minutes, 3500g centrifuged for 30 minutes, the supernatant was human serum. The serum was mixed with 100mM β mercaptoethanol uniformly and treated by boiling water bath for 10 minutes, 12000g high speed centrifuged for 10 minutes after cooling, the supernatant and 1/5 of 5x loading buffer solution by volume were mixed. The fresh CSF was obtained from hospital and placed in ice box for transportation, treated on the day. The fresh CSF was mixed with 5x loading buffer directly and made into samples directly for loading.

2. Western blotting, 15μL prepared loading sample was put in the loading well, the protein was separated with 4-12% prefabricated gel (Life technology C#NP0321BOX) and moved to PVDF membrane. The membrane was subjected to primary antibody (Rabbit-anti-sDSS1) (antigen sequence: C-terminal 31 amino acid sequences of sDSS1 protein) incubation, PBST solution cleaning three times; and secondary antibody (Goat-anti-rabbit HRP antibody) incubation. It was cleaned with PBST three times, developed by luminescent liquid and the bands were displayed by X-ray film.

3. Cell culture, the human glioma cells (U87-MG cells) were bought from ATCC, the cells were cultured in complete cell culture medium containing 90% basal medium DMEM and 10% FBS, cultured in cell incubator (temperature 37℃, humidity 95%, CO2 concentration 5%), subcultured once every two days.

4. PCR experiment, the U87-MG cells were collected and lysed rapidly, the total RNA was extracted from cell lysis solution by using a total RNA extraction kit (QIAGEN,51304), the RNA sample was treated with 1U/μL DNase I at room temperature for 15 minutes to remove residual genome DNA. The obtained RNA sample was all converted and synthesized into cDNA by using a cDNA synthesis kit (TransGen Biotech, AT301) and used as template sample for subsequent PCR experiment. 20μL reaction system was used in the PCR reaction, including 10μL

PCR premixed reagent (PCRTaq Mixture) (Omega bio-tek, TQ2200), 0.5μL cDNA template (3.5μg/mL), 0.5μL primer, 9μL ultrapure water, mixed uniformly before PCR reaction. DSS1 cDNA primers: forward primer: GCAGACAGTCGAGATGTCAGAG, reverse primer: TTCTTCTGGATGCTATGAAGTCTCC; sDSS1 cDNA primers: forward primer: GCAGACAGTCGAGATGTCAGAG, reverse primer: TGATGATCTGTTAACAGCAGAGG. PCR reaction procedure: 94℃ 10 minutes, cyclic reaction 40 times: including 94℃ 10s, 62℃ 20s, 72℃ 20s, 72℃ 10 minutes after the circulation is finished, stored at 4℃ and the DNA content in PCR product was tested by 3% sepharose [0.05% SYBR Green Stain (Thermo Fisher, 4472903)] electrophoresis.

Result analysis:

The signal of sDSS1 protein can be detected in CSF or serum by using the specific antibody of sDSS1. The serum samples derived from different individuals manifest different signal modes, the sDSS1 signal in the serum of the individual after exercise was apparently higher than that of the individual in resting state (Fig.3A). The mRNA signal of sDSS1 gene can be detected in U87-MG cells, the gene sequencing result of PCR amplified product was identical to the sequence of database (Fig.3B). These results show that the sDSS1 protein is a sort of protein, existing in CSF and serum.

Example 3, preparation of a small amount of sDSS1 protein

Experimental method

1. SDSS1 protein preparation: the nucleotide segment of total gene synthesis coded human sDSS1 protein (see SEQ ID NO: 17) was inserted into the back of Hisx6-V5 tag in pET151D. The plasmid was transferred to the expression strain BL21 (DE3). The Escherichia coli was fused with expression Hisx6-V5-sDSS1 protein, the Ni-NTA gel column was used for preliminary purification, and then the SDS-PAGE was used for gel purification. The cut strip containing

His-V5-sDSS1 protein was put in the bag filter with transfer buffer. The protein was removed from the gel under the drive of electric field and collected in the bag filter. The protein was concentrated to about 500 μL, dialyzed in PBS solution at 4℃ four times, 200 ml each time.

2. SDS polyacrylamide gel electrophoresis, the purified sDSS1 protein or bacterial lysis solution protein was mixed with 5x loading buffer solution, treated by boiling water bath for 10 minutes, 12000g high speed centrifuged for 10 minutes, the supernatant was extracted for analysis. The protein was separated by 4-12% prefabricated gel, the gel was stained for 1 hour using Coomassie brilliant blue staining solution, and decolored by destainer at room temperature over night. When the decolorization was completed, the bands on the gel were observed and photographed.

Result analysis:

The positive cloned Escherichia coli strain was selected, the culture was expanded, the bacterial cells were stimulated by IPTG to express objective protein at the beginning of logarithmic phase of bacterial growth. The bacteria were lysed, the objective protein expression level was tested. The result shows after the IPTG stimulation, the sDSS1 protein expression level of bacterial cells was upgraded significantly. The protein bands were obvious in the gel image (Fig.4A). After the bacterial lysis solution was preliminarily purified by Ni-NTA gel column, the objective protein in concentrate was concentrated greatly (channel *b*), the impure protein content decreased, very pure sDSS1 protein could be obtained by further purification (channel *a*)(Fig.4B), applicable to subsequent bioexperiment. The purified sDSS1 protein was quantified by BCA protein, the final concentration was 0.72mg/ml, stored at 4℃ for future use.

Example 4, sDSS1 protein reacts with oxidation protein and shields cytotoxicity of oxidation protein

Experimental method

1. Reaction between oxidized serum and sDSS1 protein, 3500g of fresh blood was centrifuged for 30 minutes, the upper serum was extracted for subsequent experiment. The 10μL sDSS1 protein solution (0.72mg/mL) was mixed with 10, 20, 50 and 100μL oxidized serums respectively, the mass ratios of sDSS1 to serum protein were about 1:100, 1:200, 1:500 and 1:1000, mixed with 20 μ M Fenton reagent ($FeSO_4$ and H_2O_2 were mixed as per mass ratio of 1:1), incubated in a dark place at 4℃ over night. On the next day, the reacting His-V5-sDSS1 was separated by using 10 μ L Ni-NTA beads. The reactant liquor was mixed with the beads at 4℃ for 2 hours, the magnetic separation device adsorbed the beads on the tube wall, the liquid was removed, 1ml PBST was applied, the tube was removed from the magnetic separation device, after repeated oscillation cleaning, the magnetic separation device adsorbed the beads, the PBST was sucked away, and the above steps were repeated four times. Finally, the protein was eluted with 50μL TBS containing 50mM EDTA, the eluent was mixed with isometric 2x SDS solution, treated at 100℃ for 10 minutes, 12000g centrifuged for 10 minutes, the supernatant was extracted for test. The supernatant was mixed with 5X loading buffer solution, heated at 100℃ for 10 minutes, the prepared sample was used for western blotting.

2. Preparation of oxidized FBS and AOPP, 10mL FBS was mixed with 10mM NaClO and treated for 1 hour, the oxidized serum was dialyzed continuously in PBS solution using 3000Da bag filter for 24 hours, the solution was changed at intervals of 8 hours during dialysis, the treated serum solution was mixed with 1mM vitamin C (Vc) to remove the participant oxidizer completely. The protein concentration was tested by BCA protein quantification. The content of oxidation protein was tested by using two methods, the dityrosine value in the oxidized serum measured by chloramine-T was 75.31μM/mg protein (untreated serum was 15.05 μmol/mg protein), the carbonyl content detected by dinitrophenylhydrazine was 16.33nmol/mg protein (untreated serum was 13.68 nmol/mg protein).

The 10mg serum albumin was treated with 160mM NaClO for 1 hour, the oxidation protein was dialyzed continuously in PBS solution using 3000Da bag filter for 24 hours, the solution was changed at intervals of 8 hours during dialysis. The protein concentration of the treated AOPP was determined by BCA protein quantification. The dityrosine value of AOPP sample measured by chloramine-T was 54.21μmol/mg protein (untreated BSA was 14.55μmol/mg protein), the carbonyl content measured by dinitrophenylhydrazine was 1042.57nmol/mg protein (untreated BSA was 10.26 nmol/mg protein).

3. Reaction between AOPP and sDSS1 protein, the 150μL reaction system was mixed with 30μg AOPP protein (200μg/mL), and mixed with 15μg (100μg/mL), 30μg (200μg/mL) and 60μg (400μg/mL) sDSS1 protein respectively, the excess volume was supplemented by aseptic PBS solution. The solution was stirred uniformly and reacted at 4℃ over night. The sample after reaction was mixed with 5x loading buffer solution, heated at 100℃ for 10 minutes, the treated sample was separated by SDS-PAGE and the bands were displayed by Coomassie brilliant blue staining.

4. Western blotting, the protein mixture after reaction was mixed with 5x loading buffer, treated by boiling water bath for 10 minutes for western blotting analysis. The specific method was the same as described above. The antibody was V5-HRP antibody (1:5000 diluted).

5. Cell line culture, the human neuroblastoma cells (SH-SY5Y) were grown in the basal medium DMEM with 10% FBS; the cells were subcultured once every two days.

6. Cell viability test, in order to test the effect of sDSS1 protein on the cytotoxicity of oxidized serum, the SH-SY5Y cells were inoculated to a 96-well plate as per 10^4 cells per well, 200μL complete medium. 12 hours later, the complete medium was changed to DMEM without serum containing 0.5%BSA 200μL per well. After 24 hours of treatment, the DMEM solution was changed to

10% oxidized serum and 10% oxidized serum containing 20μg/mL sDSS1 protein as culture medium, 200μL per well. After 48 hours of treatment, the old culture medium was removed from the 96-well plate, 100μL diluted CCK-8 working fluid (1:20 diluted) (DOJINDO, CK04) was put in each well to test the changes in cell viability. The group with BSA at isoconcentration was the control group.

In order to test the protective effect of sDSS1 protein on the cytotoxicity induced by AOPP, the SH-SY5Y cells were inoculated to the 96-well plate as per $2X10^4$ cells per well, after 12 hours of adhesion, the culture medium was changed to culture medium without serum containing 0.5%BSA. After 24 hours of treatment, it was changed to culture medium without serum containing 100μg/mL AOPP protein, and the treatment group was provided with 100μg/mL sDSS1 protein, 200μL per well. After 48 hours of treatment of 96-well plate, the changes in cell viability were tested by using CCK-8 kit.

Result analysis:

The serum contained a lot of proteins, mainly being serum albumin. Under the effect of Fenton reagent, the proteins in serum were oxidized, the oxidation products reacted with sDSS1 to form complexes. In control group, the sDSS1 protein monomer had no obvious protein aggregation. In the experimental group, the co-incubation with serum led to the formation of lots of high molecular weight protein complexes, these complexes cannot be separated by SDS-PAGE (Fig. 5A). The result shows that the sDSS1 protein can combine with the oxidation protein in serum. In the cytotoxicity experiment, compared with control serum, the addition of 10% oxidation protein could depress cell proliferation and cell viability obviously, and 20μg/mL sDSS1 protein in the culture medium could retrieve the cytotoxicity of oxidation protein (Fig. 5B).

The sDSS1 was mixed with AOPP, the sDSS1 and AOPP were combined to form complexes, these complexes cannot be separated by SDS-PAGE. The number of complexes increased apparently with the sDSS1 protein concentration in the

reaction system (Fig. 5C). In the cell experiment, the AOPP had significant cytotoxicity for cells, reducing the cell viability, and the sDSS1 at isoconcentration could shield the cytotoxicity of AOPP completely (Fig. 5D). To sum up the results, the sDSS1 can protect the cells from the cytotoxicity of oxidized serum or AOPP.

In addition, to sum up the reaction between sDSS1 protein and oxidation protein, two proteins can combine with the oxidation protein tightly, this binding force can resist high concentration of SDS, which seems to be covalent interaction. The difference is that the combination process of DSS1 and oxidation protein needs the assistance of an ATP enzyme [Zhang et al, 2014]. Our evidence shows that the tight coupling of sDSS1 and oxidation protein is free of ATP, the ATP enzyme is not required. According to the amino acid sequences of DSS1 and sDSS1, the sequences of amino acid positions 1 to 58 of the two proteins are identical, and the sequence of amino acid positions 59 to 70 of DSS1 are completely different from the sequence of amino acid positions 59 to 89 of sDSS1. The tight coupling of DSS1 and sDSS1 with oxidation protein is supposed to be derived from the shared amino acid sequences, i.e. the sequence of the first 58 amino acids. The difference in characteristic between sDSS1 and DSS1, which is the characteristic that the tight coupling with oxidation protein is free of ATP enzyme mediation, should be derived from the unique amino acid sequence of sDSS1, i.e. C-terminal amino acid sequence of positions 59 to 89. Altogether, the tight coupling with oxidation protein without ATP enzyme mediation of sDSS1 is derived from the organic combination of the sequences of the first 58 amino acids and the last 31 amino acids.

Example 5, sDSS1 protein reduces the formation of Aβ oligomer and reduces the cytotoxicity of Aβ oligomer

Experimental method

1. Cell line culture, the human neuroblastoma cells (SH-SY5Y) were grown in the basal medium DMEM with 10% FBS; the cells were subcultured once every two days.

2. Neural stem cell culture, the neural stem cells (NSCs) were from P2 mouse brain tissue, the NSCs of primary suspension culture were used for toxicity test after two subcultures, the NSCs were cultured in the stem cell culture medium, including 88% DMEM/F12 basal medium (Gibco, C#12500-062), 10% Proliferation supplementary additive (Stem cell technology, C#05701), 2%BSA (Sigma, C#V900933), 10ng/mL Heparin (Sigma, C#H3149), 10ng/mL bFGF (Roche, C#11104616001), 20ng/mL EGF (BD Bioscience, C#354010).

3. Reaction between Aβ and sDSS1 proteins, the Aβ protein (Human, 1-42) freeze-dried powder was supplied from Suzhou Qiangyao Biotechnology Co., Ltd. 2mg Aβ freeze-dried powder was dissolved by 20μL DMSO, diluted with PBS to 2mg/mL, stored at -20℃. The reaction system was provided with 300 μL PBS solution the 10μg Aβ and sDSS1 proteins were mixed as per molar mass ratios 1:1, 1:5 and 1:10, and then incubated at 4℃ over night. The incubated reactant was mixed with 5X loading buffer solution, treated at 100℃ for 10 minutes for western blotting analysis.

4. Aβ protein pretreatment, the Aβ stock solution was diluted with basal medium (pH7.2) to 1000μg/mL, the Aβ diluent was incubated at 4℃ for 24 hours to form oligomer for cell experiment. The Aβ concentration in subsequent experiment was always labeled according to the protein concentration before incubation.

5. Western blotting, the treated reactant was separated by SDS-PAGE for western blotting analysis, the specific method was the same as described above. The antibodies used were V5-HRP antibody (1:5000 diluted), Aβ antibody (Cell signal technology, 9888), secondary antibody (Goat-anti-rabbit HRP antibody).

6. Cell viability test, the SH-SY5Y cells were inoculated to the 96-well plate as per $2x10^4$ cells per well, after 12 hours of adhesion, the old culture medium was changed to culture medium without serum containing 0.5%BSA, after 24 hours of treatment, the old culture medium was changed to DMEM solution containing Aβ or Aβ and sDSS1 proteins. After 48 hours of treatment of cells, the cell viability level was tested by CCK-8 kit.

7. Cell apoptosis test, the cell apoptosis test kit was bought from DOJINDO chemical technology (Shanghai) corp. (AD10). The SH-SY5Y cells were inoculated to the 6-well plate as per $3x10^5$ cells per well, after 12 hours of adhesion, the old culture medium was changed to DMEM solution without serum containing 0.5%BSA. After 24 hours of treatment, it was changed to solution containing Aβ or Aβ and sDSS1 proteins. After 48 hours of treatment, the cell apoptosis level was tested by apoptosis kit. All of the solution and cells were collected, the supernatant was removed by centrifugation. The cells were resuspended in the 400μL staining buffer solution provided by the apoptosis kit, 185μL cell suspension was extracted for subsequent test. The cell suspension was mixed with 5 μ L Annexin V staining solution uniformly, the cells were incubated at 37℃ for 10 minutes. It was mixed with 10 μ L PI staining solution uniformly, the cell apoptosis level was tested by flow cytometer.

The NSCs were firstly adhered and cultured in the 6-well plate, the plate was treated with 0.025% Laminin for at least two hours, and cleaned with aseptic PBS 6 times for future use. The NSCs were made into unicells and inoculated to 6-well plate as per $3x10^5$ per well, the cells were adhered for 24h for subsequent experiment.

Result analysis

The Aβ protein had obvious aggregation after incubation, there were protein aggregates of different sizes formed within 10-20KD. According to previous reports, these Aβ oligomers were the main source of the Aβ induced cytotoxicity.

After co-incubation of sDSS1 protein and Aβ, the sDSS1 protein and Aβ protein aggregated to form high molecular weight complex (molecular weight higher than 20KD), the oligomers formed withtin 10-20KD were reduced obviously (Fig. 6A). As the sDSS1 protein concentration increased, the formation of Aβ oligomer was depressed apparently. According to the sDSS1 protein signal detection, the complex was formed by the reaction between sDSS1 protein and Aβ (Fig. 6B), and it could not be separated by SDS-PAGE.

The shielding effect of sDSS1 protein on the Aβ induced cytotoxicity was tested. In the cell viability test, the cell viability declined significantly after the pretreated Aβ oligomer was applied. When the culture medium was mixed with sDSS1 protein, the SH-SY5Y cell viability was recovered significantly and the cell viability was higher than control group (Fig. 6C). In the cell apoptosis test, the addition of Aβ oligomer to the culture medium induced the apoptosis of SH-SY5Y cells or NSCs. The addition of sDSS1 protein to the culture medium can reduce the early apoptosis and late apoptosis levels of cells significantly (Fig. 6D, Fig. 6E). According to the results, the sDSS1 protein can combine with Aβ protein to reduce the Aβ oligomer formation, so as to mitigate the cytotoxicity induced by Aβ protein.

Example 6 sDSS1 protein reduces cytotoxicity of amylin oligomer

Experimental method

1. Cell line culture, the human neuroblastoma cells (SH-SY5Y) were grown in the basal medium DMEM with 10% FBS; the cells were subcultured once every two days.

2. Amylin protein pretreatment, the amylin protein (Human) freeze-dried powder was supplied from Suzhou Qiangyao Biotechnology Corp. The 2mg amylin freeze-dried powder was dissolved to 2mg/mL in 10mM sodium acetate solution (pH5.5), stored at -20℃. The amylin stock solution was diluted to 1mg/mL with basal medium (pH7.2). The amylin diluent was incubated at 4℃ for

48 hours to form oligomer for cell experiment. The amylin concentration in subsequent experiment was always labeled according to the protein concentration before incubation.

3. Cell viability test, the SH-SY5Y cells were inoculated to 96-well plate as per $2x10^4$ cells per well, after 12 hours of adhesion, the old culture medium was changed to culture medium without serum containing 0.5%BSA. After 24 hours of treatment, the old culture medium was removed and the DMEM solution containing amylin or amylin and sDSS1 proteins was applied. After 48 hours of treatment of cells, the cell viability level was tested by CCK-8 kit.

Result analysis

The addition of 10μM inbubated amylin protein to the cell culture medium can induce significant cytotoxicity, and the addition of sDSS1 protein can shield the cytotoxicity induced by amylin oligomer, the cell viability was even higher than control group (Fig.7), meaning the sDSS1 protein can shield the cytotoxicity of amylin protein effectively.

Example 7 sDSS1 protein reduces cytotoxicity of glycosylated protein

Experimental method

1. Cell line culture, the human neuroblastoma cells (SH-SY5Y) were grown in the basal medium DMEM with 10% FBS; the cells were subcultured once every two days.

2. Glycosylated protein preparation, 10mg/mL serum albumin and 2.5M ribose were mixed and incubated at 37℃ for 7 days, and then dialyzed in PBS using 3000Da bag filter for 24 hours, the solution was changed at intervals of 8 hours. The completed glycosylated protein was quantified by BCA, the sample was stored at -80℃ for future use.

3. Cell viability test, the SH-SY5Y cells were inoculated to 96-well plate as per $2x10^4$ cells per well, after 12 hours of adhesion, the old culture medium was

changed to culture medium without serum containing 0.5%BSA. After 24 hours of treatment, the old culture medium was removed and the DMEM solution containing glycosylated protein or glycosylated protein and sDSS1 proteins at different concentrations was applied, the group with BSA at isoconcentration was used for control. After 48 hours of treatment of cells, the cell viability level was tested by CCK-8 kit.

Result analysis

The addition of 400μg/mL glycosylated protein to the cell culture medium can induce significant cytotoxicity, and the addition of sDSS1 protein can reduce the cytotoxicity induced by glycosylated protein. As the sDSS1 protein concentration increased, the cell viability was even higher than control group (Fig.8), meaning the sDSS1 protein can shield the cytotoxicity of glycosylated protein effectively.

Example 8, sDSS1 protein prolongs postoperative survival time of senescence-accelerated mouse SAMP

Experimental method

1. Animal feeding, the senescence-accelerated SAMP8 mice (5 months old, male) were bought from Beijing Vital River Laboratory Animal Technology Co., Ltd., the animals were fed at the clean laboratory animal breeding center of Southern Model Organism Center. The animals were provided with sufficient aseptic water and standard mouse breeding feed, 12h/12h dark-and-bright alternate illumination, the bedding and cage were changed monthly, the animal survival was observed daily.

2. Adenovirus synthesis, the nucleic acid sequence (see SEQ ID NO: 17) of adenovirus expressing sDSS1 protein was provided by us (inventors), the adenovirus construction and synthesis were completed by Cyagen (Guangzhou) Biotechnology Co., Ltd. The adenovirus promoter and transcription region sequence composition: pAV[Exp]-UBC>EGFP:T2A:ORF_363bp, including

ubiquitin protein promoter sequence. According to determination, the virus titer was larger than 10^{10}PFU/mL. According to the validation by infecting U87-MG cells and mouse neuroblastoma cells (N2a), the adenovirus can infect cells and express sDSS1 protein efficiently. The adenovirus expressing GFP protein (pAV[Exp]-UBC>EGFP) was used as control.

3. Stereotactic injection, the senescence-accelerated SAMP8 mouse (6 months old, male) was anaesthetized by intraperitoneal injection with 20% urethane (dissolved in normal saline, impurities and bacteria removed by 0.22μm filter) as per 800mg/kg body weight. When the mouse was anaesthetized, it was fixed to the mouse stereotaxic apparatus (Stoelting, 51500), the cranial bone was kept horizontal. The head skin was incised to expose the cranial bone, the bregma was taken as the starting coordinate to locate the lateral ventricle region (0.58mm, 1.25mm, 1.75mm). The marking point was perforated by dental drill. 2μL of virus liquid was sucked by the microinjector (Hamilton 600-2.5μL, syringe needle diameter 0.2mm), the lateral ventricle region was relocated according to the same coordinate values. The needle was inserted into the lateral ventricle region quickly according to the specified coordinate values, the virus liquid was injected slowly at 200nL/min on average, for a total amount of 1000nL. After the injection was done, each time the needle was lifted for 0.25mm, it was waited for 3 minutes until the needle was drawn out of the brain tissue completely. The lateral ventricle region on the opposite side was relocated, the needle insertion, injection and needle lifting were completed, 1μL virus liquid was injected. For the mouse after injection, the cranial bone and peripheral tissues were wiped with 100U/mL ampicillin/streptomycin, and the skin was sutured. The abdominal cavity of the mouse was injected with 150μL antibiotics, and the mouse was put in the cage with abdomen up until the mouse was awake.

Result analysis

The schematic diagram indicates the basic operation of injecting virus into the mouse's lateral ventricle and the adenovirus injection site (Fig.9A). Wherein

there were two batches of mice injected with adenovirus expressing sDSS1, 10 mice in total; there were two batches of mice injected with adenovirus expressing GFP, 14 mice in total. Within 1 month after the injection of adenovirus, three mice of the control group died, and one mouse of the experimental group died. Within 8 months, the mice of the control group died successively, whereas only one mouse of the experimental group died (Fig.9B). To sum up the results, the postoperative survival time of the SAMP8 mice injected with adenovirus expressing sDSS1 protein was significantly longer than that of the mice only injected with control virus. The significant difference was analyzed by ANOVA (P-value<0.01).

The above detailed description only specifies the feasible embodiments of the present application, and is not intended to limit the scope of protection of the present application. Any equivalent embodiments or alterations not deviating from the gist of the present application shall be covered in the scope of protection of the present application.

CLAIMS

What is claimed is:

1. A secretory deleted split hand/split foot 1 (sDSS1) protein for use in therapy.

2. The sDSS1 protein for use according to claim 1, wherein said sDSS1 protein comprises an amino acid sequence set forth in SEQ ID NO: 1, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16.

3. Use of a secretory deleted split hand/split foot 1 (sDSS1) protein in the manufacture of a medicament for reducing apoptosis or promoting cell survival of a live cell that is exposed to a pathogenic polypeptide.

4. The use of claim 3, wherein said sDSS1 protein comprises an amino acid sequence set forth in SEQ ID NO: 1, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16.

5. The use of claim 3 or 4, wherein said pathogenic polypeptide is selected from advanced oxidation protein products (AOPP), amyloid-β, amylin, and glycosylated protein.

6. The use of any one of claims 3-5, wherein said pathogenic polypeptide is advanced oxidation protein products (AOPP).

7. The use of any one of claims 3-5, wherein said pathogenic polypeptide is amyloid-β.

8. The use of any one of claims 3-5, wherein said pathogenic polypeptide is amylin.

9. The use of any one of claims 3-5, wherein said pathogenic polypeptide is glycosylated protein.

10. The use of any one of claims 3-5, wherein said pathogenic polypeptide is glycated protein.

11. The sDSS1 protein for use according to claims 1 or 2 or the use of any one of claims 3 to 10, wherein said sDSS1 protein comprises an amino acid sequence set forth in SEQ ID NO: 1.

12. The sDSS1 protein for use or the use according to claim 11, wherein the sDSS1 protein is encoded by a nucleotide.

13. The sDSS1 protein for use or the use according to claim 12, wherein the nucleotide comprises DNA or RNA.