CD99 as target/marker for insulin resistance

Type 2 diabetes is a disease of fast growing worldwide importance and can be described as a failure of the pancreatic beta-cell (beta-cell failure) to compensate, with enhanced insulin secretion of the beta-cells, for peripheral insulin resistance.

Insulin resistance can be considered the first step in the development of Type 2 Diabetes and develops years before diabetes is diagnosed. During this first stage, patients remain normoglycaemic and compensate for reduced insulin responsiveness of muscle and liver by an enhanced secretion of insulin. At later stages in the development of Type 2 Diabetes, beta cell function decreases, leading to impaired glucose tolerance and, finally, diabetes. Early intervention by either weight loss, exercise, or pharmaceutical treatment, was shown to delay or even prevent the development of diabetes in patients with impaired glucose tolerance (Diabetes Prevention Program Research Group, N. Engl. J. Med. 346 (2002) 393-403). Therefore, an early diagnosis of insulin resistance would allow early intervention by anti-diabetic treatment or other measures that would prevent progression of the disease. To date, the only reliable possibility to detect insulin resistance is by the euglycemic-hyperinsulinemic clamp (EHC). HOMA modeling is often used for assessing insulin resistance but is not an accepted diagnostic method (Wallace et al., Diabetes Care 27(2004)1487ff.). Due to them being time consuming and labor intensive, these methods do not lend themselves to broad patient screening programs. A molecular marker for insulin resistance would therefore be extremely useful for the detection of this condition.

Most currently used Type 2 Diabetes treatments do not directly address Insulin resistance. Safety concerns exist for those that do primarily act at the level of peripheral glucose uptake (e.g. insulin sensitizers). Therefore, it would also be useful to identify additional, better targets for treatment and markers for detection of Insulin Resistance or efficacy that are more sensitive or more reliable than the markers commonly used, such as the EHC or HOMA method.

Furthermore, it would be an advantage to identify markers that can be detected in plasma.

The aim of the present invention is to identify and provide a novel target to screen for compounds that prevent, attenuate, or inhibit Insulin Resistance, and for a marker that allows for monitoring and/or diagnosis of Insulin Resistance at an earlier stage of type II diabetes and more reliably than can presently be done.

Surprisingly, it was found that the use of protein CD99 can overcome, at least in part, the problems known from the state of the art.

CD99 is a 32-kDa transmembrane protein which has been implicated in migration of monocytes, and differentiation and apotosis of T- cells and thymocytes. The biologic function of CD99 is poorly understood.

Surprisingly, it was found that changes in the levels of secreted CD99 are found in Insulin Resistance. Therefore, the present invention provides a target for the treatment and/or prevention of Insulin Resistance, and a novel marker for the early diagnosis of Insulin Resistance in diabetes. Preferably, said changes are an increase in the levels of secreted CD99.

The fact that Endoglin is a transmembrane protein implies that in order for a secreted form to appear in plasma, fragments of the sequence of Seq ID No. 1 have to be generated. Thus, the target used for the methods of the present invention, or the markers detectable by the methods of the present invention, also includes soluble fragments of Seq ID No. 1. Such soluble fragments may comprise either the portion or any fragment thereof of the protein from the N-terminus of the mature sequence to the amino acid preceding the transmembrane domain, or the cytoplasmic domain (the first amino acid following the transmembrane domain to the C-terminus of the sequence). Therefore, the term "CD99" and "protein CD99", as used herein, are understood to include soluble fragments of Seq ID No. 1 as well as the protein of Seq ID No. 1 or mutants thereof which are at least 90 % homologous to Seq ID No. 1. To determine the percent homology or identity of two amino acid sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in the sequence of one polypeptide for optimal alignment with the other polypeptide or nucleic acid molecule). The amino acid residues or nucleotides at corresponding amino acid positions are then compared. When a position in one sequence is occupied by the same amino acid residue as the corresponding position in the other sequence, then the molecules are homologous at that

position. As used herein, amino acid "homology" is equivalent to amino acid "identity". The percent homology between the two sequences is a function of the number of identical positions shared by the sequences *(i.e.,* percent homology equals the number of identical positions/total number of positions times 100).

In a preferred embodiment, the marker CD99 consists of any fragment or mutant or native form of Seq ID No. 1 which can be detected by the ELISA described in example 4.

In preferred embodiments, the novel target and/or marker CD99 may be used for diagnostic, monitoring as well as for screening purposes.

When used in patient monitoring, the diagnostic method according to the present invention may help to assess efficacy of treatment and recurrence of Insulin Resistance in the follow-up of patients. Therefore, the present invention provides the use of protein CD99 for monitoring the efficacy of treatment of diabetes.

In a preferred embodiment, the diagnostic method according to the present invention is used for patient screening purposes. I.e., it is used to assess subjects without a prior diagnosis of diabetes by measuring the level of CD99 and correlating the level of CD99 to the presence or absence of Insulin Resistance.

The methods of the present invention are useful for monitoring progression of the disease through the different stages leading to diabetes, namely Insulin Resistance, Impaired Glucose Tolerance and Diabetes.

The present invention thus provides a method for monitoring the progression of diabetes, comprising the steps of (a) providing a liquid sample obtained from an individual, (b) contacting said sample with a specific binding agent for CD99 under conditions appropriate for formation of a complex between said binding agent and CD99, and (c) correlating the amount of complex formed in (b) to the amount of complex formed in Insulin Resistance.

The present invention also provides a method for monitoring the efficacy of treatment of diabetes, comprising the steps of (a) providing a liquid sample obtained from a patient treated against diabetes, (b) contacting said sample with a specific binding agent for CD99 under conditions appropriate for formation of a complex between said

binding agent and CD99, and (c) correlating the amount of complex formed in (b) to the amount of complex formed in the absence of treatment.

The present invention provides a method of screening for a compound which interacts with CD99, comprising the steps of a) contacting protein CD99 with a compound or a plurality of compounds under compositions which allow interaction of said compound or a plurality of compounds with CD99; and b) detecting the interaction between said compound or plurality of compounds with said polypeptide.

The present invention provides a method of screening for a compound that prevents and/or inhibits and/or attenuates Insulin Resistance, comprising the steps of a) contacting a compound with protein CD99; and b) measuring the activity of protein CD99; wherein a compound which inhibits or stimulates the activity of protein CD99 is a compound that may prevent and/or inhibit and/or attenuate Insulin Resistance. Preferably, said method additionally comprises the step of immobilizing protein CD99 prior to step a) or between steps a) and b).

The term „activity" as used herein relates e.g. to the ability of CD99 to mediate migration of cells, to induce apoptosis or to bind to cyclophilin A (see e.g. Cerisano et al., 2004, Oncogene 23, 5664-5674; Kim et al., 2004, Immunol. Lett. 95, 155-159).

The present invention also includes cell-free assays. Such assays involve contacting a form of CD99 (e.g., full-length polypeptide, a biologically active fragment of said polypeptide, or a fusion protein comprising all or a portion of said polypeptide) with a test compound and determining the ability of the test compound to bind to said polypeptide. Binding of the test compound to said polypeptide can be determined either directly or indirectly as described above. In one embodiment, the assay includes contacting the said polypeptide with a known compound which binds said polypeptide to form an assay mixture, contacting the assay mixture with a test compound, and determining the ability of the test compound to interact with said polypeptide, wherein determining the ability of the test compound to interact with said polypeptide comprises determining the ability of the test compound to preferentially bind to the said polypeptide as compared to the known compound.

The cell-free assays of the present invention are amenable to use of either a membrane-bound form of a polypeptide or a soluble fragment thereof. In the case of cell-free assays comprising the membrane-bound form of the polypeptide, it may be

desirable to utilize a solubilizing agent such that the membrane-bound form of the polypeptide is maintained in solution. Examples of such solubilizing agents include non-ionic detergents such as n-octylglucoside, n-dodecylglucoside, n-dodecylmaltoside, octanoyl-N-methylglucamide, decanoyl-N-methylglucamide, Triton X-100, Triton X-114, Thesit, Isotridecypoly(ethylene glycol ether)n, 3-[(3-cholamidopropyl)dimethylamminio]-1-propane sulfonate (CHAPS), 3-[(3-cholamidopropyl)dimethylamminio]-2-hydroxy-1-propane sulfonate (CHAPSO), or N-dodecyl-N, N-dimethyl-3-ammonio-1 -propane sulfonate.

In various embodiments of the above assay methods of the present invention, it may be desirable to immobilize a polypeptide to facilitate separation of complexed from uncomplexed forms of the polypeptide with a binding molecule, as well as to accommodate automation of the assay. Binding of a test compound to a polypeptide, or interaction of a polypeptide with a binding molecule in the presence and absence of a candidate compound, can be accomplished in any vessel suitable for containing the reactants. Examples of such vessels include microtitre plates, test tubes, and micro-centrifuge tubes. In one embodiment, a fusion protein can be provided which adds a domain that allows one or both of the proteins to be bound to a matrix. For example, glutathione-S-transferase fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical; St. Louis, Mo.) or glutathione derivatized microtitre plates, which are then combined with the test compound or the test compound and either the non-adsorbed binding protein or polypeptide, and the mixture incubated under conditions conducive to complex formation (e.g., at physiological conditions for salt and pH). Following incubation, the beads or microtitre plate wells are washed to remove any unbound components and complex formation is measured either directly or indirectly, for example, as described above. Alternatively, the complexes can be dissociated from the matrix, and the level of binding or activity of a polypeptide hereinbefore described can be determined using standard techniques.

Other techniques for immobilizing proteins on matrices can also be used in the screening assays of the invention. For example, either a polypeptide hereinbefore described or its binding molecule can be immobilized utilizing conjugation of biotin and streptavidin. Biotinylated polypeptide of the invention or target molecules can be prepared from biotin-NHS (N-hydroxy-succinimide) using techniques well known in the art (e.g., biotinylation kit, Pierce Chemicals; Rockford, Ill.), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). Alternatively, antibodies reactive with a polypeptide or binding molecules, but which do not interfere with binding of the

polypeptide of the invention to its binding molecule; can be derivatized to the wells of the plate. Unbound binding protein or polypeptide of the invention is trapped in the wells by antibody conjugation. Methods for detecting such complexes, in addition to those described above for the GST-immobilized complexes, include immunodetection of complexes using antibodies reactive with a polypeptide hereinbefore described or binding molecule, as well as enzyme-linked assays which rely on detecting an enzymatic activity associated with a polypeptide or binding molecule.

The present invention also provides a method of screening for a compound that prevents and/or inhibits and/or delays Insulin Resistance, comprising the step of detecting soluble CD99 secreted from a host in the presence or absence of said compound, wherein a compound that prevents and/or inhibits and/or delays Insulin Resistance is a compound with which the level of CD99 secreted from a host is changed.

A host may be a model cell representing beta-cells in culture, or an animal which can be used as a model for Insulin Resistance.

The present invention also provides for a use of protein CD99 as a target and/or as a marker for screening for a compound that prevents and/or inhibits Insulin Resistance.

The diagnostic, monitoring or patient screening methods according to the present invention are based on a liquid sample which is derived from an individual. Unlike to methods known from the art CD99 is specifically measured from this liquid sample by use of a specific binding agent.

A specific binding agent is, e.g., a receptor for CD99 or an antibody to CD99. As the skilled artisan will appreciate the term specific is used to indicate that other biomolecules present in the sample do not significantly bind to the binding agent specific for CD99. A level of less than 5% cross-reactivity is considered not significant.

A specific binding agent preferably is an antibody reactive with CD99. The term antibody refers to a polyclonal antibody, a monoclonal antibody, fragments of such antibodies, as well as to genetic constructs comprising the binding domain of an antibody.

Antibodies are generated by state of the art procedures, e.g., as described in Tijssen (Tijssen, P., Practice and theory of enzyme immunoassays 11 (1990) the whole

book, especially pages 43-78; Elsevier, Amsterdam). For the achievements as disclosed in the present invention polyclonal antibodies raised in rabbits have been used. However, clearly also polyclonal antibodies from different species, e.g. rats or guinea pigs, as well as monoclonal antibodies can also be used. Since monoclonal antibodies can be produced in any amount required with constant properties, they represent ideal tools in development of an assay for clinical routine. The generation and use of monoclonal antibodies to CD99 in a method according to the present invention is yet another preferred embodiment.

As the skilled artisan will appreciate now, that CD99 has been identified as a marker which is useful in the diagnosis of Insulin Resistance, alternative ways may be used to reach a result comparable to the achievements of the present invention. For example, alternative strategies to generate antibodies may be used. Such strategies comprise amongst others the use of synthetic peptides, representing an epitope of CD99 for immunization. Alternatively, DNA immunization also known as DNA vaccination may be used.

For measurement the liquid sample obtained from an individual is contacted with the specific binding agent for CD99 under conditions appropriate for formation of a binding agent CD99-complex. Such conditions need not be specified, since the skilled artisan without any inventive effort can easily identify such appropriate incubation conditions.

As a final step according to the methods disclosed in the present invention the amount of complex is measured and correlated to the diagnosis of Insulin Resistance or to a respective control, as hereinbefore described. As the skilled artisan will appreciate there are numerous methods to measure the amount of the specific binding agent CD99-complex all described in detail in relevant textbooks (cf., e.g., Tijssen P., supra, or Diamandis, et al., eds. (1996) Immunoassay, Academic Press, Boston).

Preferably CD99 is detected in a sandwich type assay format. In such assay a first specific binding agent is used to capture CD99 on the one side and a second specific binding agent, which is labeled to be directly or indirectly detectable, is used on the other side.

As mentioned above, it has surprisingly been found that CD99 can be measured from a liquid sample obtained from an individual sample. No tissue and no biopsy sample is required to apply the marker CD99 in the diagnosis of Insulin Resistance.

In a preferred embodiment the method according to the present invention is practiced with serum as liquid sample material.

In a further preferred embodiment the method according to the present invention is practiced with plasma as liquid sample material.

In a further preferred embodiment the method according to the present invention is practiced with whole blood as liquid sample material.

Whereas application of routine proteomics methods to tissue samples, leads to the identification of many potential marker candidates for the tissue selected, the inventors of the present invention have surprisingly been able to detect protein CD99 in a bodily fluid sample. Even more surprising they have been able to demonstrate that the presence of CD99 in such liquid sample obtained from an individual can be correlated to the diagnosis of Insulin Resistance.

Antibodies to CD99 with great advantage can be used in established procedures, e.g., to Insulin Resistance in situ, in biopsies, or in immunohistological procedures.

Preferably, an antibody to CD99 is used in a qualitative (CD99 present or absent) or quantitative (CD99 amount is determined) immunoassay.

Measuring the level of protein CD99 has proven very advantageous in the field of Insulin Resistance and diabetes. Therefore, in a further preferred embodiment, the present invention relates to use of protein CD99 as a marker molecule in the diagnosis of Insulin Resistance from a liquid sample obtained from an individual.

The term marker molecule is used to indicate that changes in the level of the analyte CD99 as measured from a bodily fluid of an individual mark the presence of Insulin Resistance.

It is preferred to use the novel marker CD99 in the early diagnosis of type II diabetes.

It is especially preferred to use the novel marker CD99 in the early diagnosis of glucose intolerance.

It is also especially preferred to use the novel marker CD99 in the monitoring of disease progression in diabetes.

The use of protein CD99 itself, represents a significant progress to the challenging field of Insulin Resistance diagnosis. Combining measurements of CD99 with other known markers for diabetes, like insulin, or with other markers of Insulin Resistance yet to be discovered, leads to further improvements. Therefore in a further preferred embodiment the present invention relates to the use of CD99 as a marker molecule for diabetes, preferably for Insulin Resistance, in combination with another marker molecule for diabetes, preferably for Insulin Resistance, in the diagnosis of diabetes, preferably of Insulin Resistance from a liquid sample obtained from an individual. Preferred selected other diabetes markers with which the measurement of Insulin Resistance may be combined are insulin, pre-insulin, and/or C-peptide.

Diagnostic reagents in the field of specific binding assays, like immunoassays, usually are best provided in the form of a kit, which comprises the specific binding agent and the auxiliary reagents required to perform the assay. The present invention therefore also relates to an immunological kit comprising at least one specific binding agent for CD99 and auxiliary reagents for measurement of CD99.

One way of assessing clinical utility of the novel marker CD99 is by measuring its levels in 17 patients that were diagnosed as being insulin resistant by measuring the glucose disposal rate with the EHC method and comparing the levels with those measured in 17 patients with demonstrated normal glucose disposal rate as determined by the same methodology. For statistical analysis, standard Student's t-test evaluation is performed with values < 0.05 being taken as significant.

Accuracy of a test can be described by its receiver-operating characteristics (ROC) (see especially Zweig, M. H., and Campbell, G., Clin. Chem. 39 (1993) 561-577). The ROC graph is a plot of all of the sensitivity/specificity pairs resulting from continuously varying the decision threshold over the entire range of data observed.

The clinical performance of a laboratory test depends on its diagnostic accuracy, or the ability to correctly classify subjects into clinically relevant subgroups. Diagnostic accuracy measures the test's ability to correctly distinguish two different conditions of the subjects investigated. Such conditions are for example health and disease.

In each case, the ROC plot depicts the overlap between the two distributions by plotting the sensitivity versus 1 - specificity for the complete range of decision thresholds. On the y-axis is sensitivity, or the true-positive fraction [defined as (number of true-positive test results) (number of true-positive + number of false-negative test results)]. This has also been referred to as positivity in the presence of a disease or condition. It is calculated solely from the affected subgroup. On the x-axis is the false-positive fraction, or 1 - specificity [defined as (number of false-positive results)/(number of true-negative + number of false-positive results)]. It is an index of specificity and is calculated entirely from the unaffected subgroup. Because the true- and false-positive fractions are calculated entirely separately, by using the test results from two different subgroups, the ROC plot is independent of the prevalence of disease in the sample. Each point on the ROC plot represents a sensitivity/-specificity pair corresponding to a particular decision threshold. A test with perfect discrimination (no overlap in the two distributions of results) has an ROC plot that passes through the upper left corner, where the true-positive fraction is 1.0, or 100% (perfect sensitivity), and the false-positive fraction is 0 (perfect specificity). The theoretical plot for a test with no discrimination (identical distributions of results for the two groups) is a 45° diagonal line from the lower left corner to the upper right corner. Most plots fall in between these two extremes. (If the ROC plot falls completely below the 45° diagonal, this is easily remedied by reversing the criterion for "positivity" from "greater than" to "less than" or vice versa.) Qualitatively, the closer the plot is to the upper left corner, the higher the overall accuracy of the test.

One convenient goal to quantify the diagnostic accuracy of a laboratory test is to express its performance by a single number. The most common global measure is the area under the ROC plot. By convention, this area is always ≥ 0.5 (if it is not, one can reverse the decision rule to make it so). Values range between 1.0 (perfect separation of the test values of the two groups) and 0.5 (no apparent distributional difference between the two groups of test values). The area does not depend only on a particular portion of the plot such as the point closest to the diagonal or the sensitivity at 90% specificity, but on the entire plot. This is a quantitative, descriptive expression of how close the ROC plot is to the perfect one (area = 1.0).

Also claimed are the methods, uses and kit substantially as hereinbefore described, especially with reference to the examples below.

The following examples, references, sequence listing and figure are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

Examples

Example 1

Identification of candidate endothelial marker proteins for insulin resistance by analysis of mRNA expression in umbilical vein endothelial cells

Culture of endothelial cells, mRNA extraction, reverse transcription, labeling and hybridization to DNA microarrays

Based on an analysis of the protein levels in plasma from patients with insulin resistance compared to plasma from controls it became evident that markers of endothelial activation can be detected at increased levels in insulin resistant individuals. Recent literature supports these findings (e.g. Meigs et al., JAMA 291 (2004) 1978 ff). Therefore, highly expressed endothelial proteins carrying a signal sequence targeting them for excretion or Type 1 membrane proteins with a large extracellular domain anchored to the membrane by a single transmembrane sequence are potential novel marker proteins for endothelial activation. In order to identify candidate proteins an mRNA expression dataset was analyzed *in silico* for extracellular proteins.

Human Vascular Endothelial Cells (HUVECs) were prepared following standard procedures. Briefly, the veins were washed free of blood before filling them with Dispase-solution (Roche, Cat. No 295 825, diluted 1:10 in DMEM). The blood vessels were closed on both ends and incubated for 30 min at 37°C. The detached endothelial cells were then collected and the umbilical vein washed once with PBS. The cells were centrifuged fro 10 min at 320 x g, resuspended in P0 Culture medium: M199 (Sigma Cat. No. M7528 + 20% Fetal Calf Serum + 1% Pen/Strep + 1% Glutamine + 100µg/ml ECGS (Sigma Cat.

No E2759) + 100µg/ml Heparin (Sigma Cat. No. H3149) + 1/500 Vol. Gentamicin (Roche Cat. No. 1 059 467). After 24 h, the erythrocytes were washed off and the medium was replaced with fresh P0 medium. Aftr incubation for another 24 h, the medium was replaced with p1 medium: M199 (Sigma Cat. No. M7528 + 20% Fetal Calf Serum + 1% Pen/Strep + 1% Glutamine + 50µg/ml ECGS (Sigma Cat. No E2759) + 100µg/ml Heparin (Sigma Cat. No. H3149).

The HUVECs was cultured in p1 medium for 48h. After 48h the cells were harvested by scraping and the total cellular RNA was extracted with RNA-Bee™. From each sample 10 µg of total cellular RNA were reverse transcribed (Invitrogen, U.S.), labeled (Ambion, U.S.) and processed by using commercial kits according to the supplier's instructions. The methods of the alkaline heat fragmentation and the following hybridization of the cDNA with the U133 A and B GeneChip arrays were standard procedure provided by the manufacturer of the microchips (Affymetrix, U.S.).

The cell intensity values of the arrays were recorded with a confocal laser scanner (Hewlett Packard, U.S.) and data were analyzed using GeneChip v3.1 software (Affymetrix, U.S.). The expression level for each gene was calculated as normalized average difference of fluorescence intensity as compared to hybridization to mismatched oligonucleotides, expressed as average difference (A.D.). This experiment was performed in triplicate in order to account for biological variation.

Identification of highly expressed extracellular proteins by in silico analysis of an mRNA expression data set

The 200 genes that showed the highest mRNA levels in the above mentioned dataset (highest A.D. values) were selected. The corresponding protein sequences were analyzed by a custom software tool that predicts signal and membrane anchor sequences in a probabilistic manner. Briefly, this software tool displays signal peptide or membrane anchor predictions for proteins from SwissProt, or predicted in the eukaryotic genomes. It is based on a set of specialized, manually curated Hidden Markov Models (HMMs) that attempt to recognize the sequence features common to signal peptides or anchors, respectively (Sean R. Eddy, HMMER 2.3.2, http://hmmer.wustl.edu). As these sequence signals cannot be reliably predicted, the "signal" and "anchor" scores that any input sequence is assigned are fed into a Support Vector Machine (SVM) in a second analysis step (Cristianini N, Shawe-Taylor J. An Introduction to Support Vector Machines and other Kernel-based Learning Methods. Cambridge University Press, Cambridge, England, 2000). The SVM was trained on a set of *bona fide* examples for both classes. On this

training set, the SVM obtained the following results on three training sets (signal – anchor – neither). The proteins predicted as extracellular ("signal" or "anchor") were further evaluated for organ specificity. A search for public domain expressed sequence tags encoding the candidate proteins was carried out and grouped according to tissue source. Only those protein were retained that were expressed in blood vessels and that did not show a strong expression in other secretory organs (e.g. liver, pancreas).

Example 2

Generation of antibodies to the Insulin Resistance marker CD99

Polyclonal antibody to the Insulin Resistance marker CD99 is generated for further use of the antibody in the measurement of serum and plasma and blood levels of CD99 by immunodetection assays, e.g. Western Blotting and ELISA.

<u>Recombinant protein expression in E. coli</u>

In order to generate antibodies to CD99, recombinant expression of the protein is performed for obtaining immunogens. The expression is done applying a combination of the RTS 100 expression system and E.coli. In a first step, the DNA sequence is analyzed and recommendations for high yield cDNA silent mutational variants and respective PCR-primer sequences are obtained using the "ProteoExpert RTS E.coli HY" system. This is a commercial web based service (www.proteoexpert.com). Using the recommended primer pairs, the "RTS 100 E. coli Linear Template Generation Set, His-tag" (Roche Diagnostics GmbH, Mannheim, Germany, Cat.No. 3186237) system to generate linear PCR templates from the cDNA and for in-vitro transcription and expression of the nucleotide sequence coding for the CD99 protein is used. For Western-blot detection and later purification, the expressed protein contains a His-tag. The best expressing variant is identified. All steps from PCR to expression and detection are carried out according to the instructions of the manufacturer. The respective PCR product, containing all necessary T7 regulatory regions (promoter, ribosomal binding site and T7 terminator) is cloned into the pBAD TOPO® vector (Invitrogen, Karlsruhe, Germany, Cat. No. K 4300/01) following the manufacturer's instructions. For expression using the T7 regulatory sequences, the construct is transformed into E. coli BL 21 (DE 3) (Studier, F.W., et al., Methods Enzymol. 185 (1990) 60-89) and the transformed bacteria are cultivated in a 1 l batch for protein expression.

Purification of His-CD99 fusion protein is done following standard procedures on a Ni-chelate column. Briefly, 1 l of bacteria culture containing the expression vector for the His-CD99 fusion protein is pelleted by centrifugation. The cell pellet is resuspended in lysis buffer, containing phosphate, pH 8.0, 7 M guanidinium chloride, imidazole and thioglycerole, followed by homogenization using an Ultra-Turrax®. Insoluble material is pelleted by high speed centrifugation and the supernatant is applied to a Ni-chelate chromatographic column. The column is washed with several bed volumes of lysis buffer followed by washes with buffer, containing phosphate, pH 8.0 and urea. Finally, bound antigen is eluted using a phosphate buffer containing SDS under acidic conditions.

Production of monoclonal antibodies against the protein CD99

a) Immunization of mice

12 week old A/J mice are initially immunized intraperitoneally with 100 µg CD99. This is followed after 6 weeks by two further intraperitoneal immunizations at monthly intervals. In this process each mouse is administered 100 µg CD99 adsorbed to aluminum hydroxide and 10^9 germs of *Bordetella pertussis*. Subsequently the last two immunizations are carried out intravenously on the 3rd and 2nd day before fusion using 100 µg CD99 in PBS buffer for each.

b) Fusion and cloning

Spleen cells of the mice immunized according to a) are fused with myeloma cells according to Galfre, G., and Milstein, C., Methods in Enzymology 73 (1981) 3-46. In this process ca. $1*10^8$ spleen cells of the immunized mouse are mixed with $2x10^7$ myeloma cells (P3X63-Ag8-653, ATCC CRL1580) and centrifuged (10 min at 300 g and 4°C.). The cells are then washed once with RPMI 1640 medium without fetal calf serum (FCS) and centrifuged again at 400 g in a 50 ml conical tube. The supernatant is discarded, the cell sediment is gently loosened by tapping, 1 ml PEG (molecular weight 4000, Merck, Darmstadt) is added and mixed by pipetting. After 1 min in a water-bath at 37°C, 5 ml RPMI 1640 without FCS is added drop-wise at room temperature within a period of 4-5 min. Afterwards 5 ml RPMI 1640 containing 10% FCS is added drop-wise within ca. 1 min, mixed thoroughly, filled to 50 ml with medium (RPMI 1640 + 10% FCS) and subsequently centrifuged for 10 min at 400 g and 4°C. The sedimented cells are taken up in RPMI 1640 medium containing 10% FCS and sown in hypoxanthine-azaserine

selection medium (100 mmol/l hypoxanthine, 1 µg/ml azaserine in RPMI 1640 + 10% FCS). Interleukin 6 at 100 U/ml is added to the medium as a growth factor. After ca. 10 days the primary cultures are tested for specific antibody. CD99-positive primary cultures are cloned in 96-well cell culture plates by means of a fluorescence activated cell sorter. In this process again interleukin 6 at 100 U/ml is added to the medium as a growth additive.

c) Immunoglobulin isolation from the cell culture supernatants

The hybridoma cells obtained are sown at a density of $1x10^5$ cells per ml in RPMI 1640 medium containing 10% FCS and proliferated for 7 days in a fermenter (Thermodux Co., Wertheim/Main, Model MCS-104XL, Order No. 144-050). On average concentrations of 100 µg monoclonal antibody per ml are obtained in the culture supernatant. Purification of this antibody from the culture supernatant is carried out by conventional methods in protein chemistry (e.g. according to Bruck, C., et al., Methods in Enzymology 121 (1986) 587-695).

<u>Generation of polyclonal antibodies</u>

a) Immunization

For immunization, a fresh emulsion of the protein solution (100 µg/ml protein CD99) and complete Freund's adjuvant at the ratio of 1:1 is prepared. Each rabbit is immunized with 1 ml of the emulsion at days 1, 7, 14 and 30, 60 and 90. Blood is drawn and resulting anti-CD99 serum used for further experiments as described in examples 3 and 4.

b) Purification of IgG (immunoglobulin G) from rabbit serum by sequential precipitation with caprylic acid and ammonium sulfate

One volume of rabbit serum is diluted with 4 volumes of acetate buffer (60 mM, pH 4.0). The pH is adjusted to 4.5 with 2 M Tris-base. Caprylic acid (25 µl/ml of diluted sample) is added drop-wise under vigorous stirring. After 30 min the sample is centrifuged (13,000 x g, 30 min, 4°C), the pellet discarded and the supernatant collected. The pH of the supernatant is adjusted to 7.5 by the addition of 2 M Tris-base and filtered (0.2 µm).
The immunoglobulin in the supernatant is precipitated under vigorous stirring by the drop-wise addition of a 4 M ammonium sulfate solution to a final concentration of 2 M.

The precipitated immunoglobulins are collected by centrifugation (8000 x g, 15 min, 4°C).

The supernatant is discarded. The pellet is dissolved in 10 mM NaH_2PO_4/NaOH, pH 7.5, 30 mM NaCl and exhaustively dialyzed. The dialysate is centrifuged (13,000 x g, 15 min, 4°C) and filtered (0.2 µm).

Biotinylation of polyclonal rabbit IgG

Polyclonal rabbit IgG is brought to 10 mg/ml in 10 mM NaH_2PO_4/NaOH, pH 7.5, 30 mM NaCl. Per ml IgG solution 50 µl Biotin -N-hydroxysuccinimide (3.6 mg/ml in DMSO) are added. After 30 min at room temperature, the sample is chromatographed on Superdex 200 (10 mM NaH_2PO_4/NaOH, pH 7.5, 30 mM NaCl). The fraction containing biotinylated IgG are collected. Monoclonal antibodies have been biotinylated according to the same procedure.

Digoxygenylation of polyclonal rabbit IgG

Polyclonal rabbit IgG is brought to 10 mg/ml in 10 mM NaH_2PO_4/NaOH, 30 mM NaCl, pH 7.5. Per ml IgG solution 50 µl digoxigenin-3-O-methylcarbonyl-ε-aminocaproic acid-N-hydroxysuccinimide ester (Roche Diagnostics, Mannheim, Germany, Cat. No. 1 333 054) (3.8 mg/ml in DMSO) are added. After 30 min at room temperature, the sample is chromatographed on Superdex® 200 (10 mM NaH_2PO_4/NaOH, pH 7.5, 30 mM NaCl). The fractions containing digoxigenylated IgG are collected. Monoclonal antibodies are labeled with digoxigenin according to the same procedure.

Example 3

Western Blot

Protein samples enriched and isolated from the medium by Heparin columns (mentioned above) are solved in sample buffer consisting of 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05 % Tween 20, 1 % SDS, and centrifuged at 12,000 g for 10 min at 4°C. The protein concentration of the supernatant is measured by Bradford using a standard curve constructed from a range of known bovine serum albumin standards. After mixing

samples with sample buffer (60 mM Tris-HCl, 2% SDS, 0.1% bromophenol blue, 25% glycerol, and 14.4 mM 2-mercaptoethanol, pH 6.8) and incubation at 70°C for 5 min, samples are separated by 12.5% homogenous ExcelGel SDS gels (Amersham Bioscience) and electro transferred onto Nitrocellulose membranes. After incubation in blocking solution (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20 and 5% non-fat dry milk), membranes are incubated with rabbit anti-rat antibody for 2 hrs at room temperature, respectively. After washing 3 times for 10 min with washing solution (0.3% Tween 20 in tris-buffered saline), membranes are incubated with a horseradish peroxidase conjugated anti-rabbit IgG (H+L), anti-mouse IgG_1 and anti-mouse IgG_{2a} (Southern Biotechnology Associates, Inc., Birmingham, AL), respectively, for 1 hr at room temperature. Membranes are washed 3 times for 10 min and antigen-antibody complexes are visualized by an enhanced chemiluminescence's reagent (Western Lightning TM, PerkinElmer Life Sciences, Inc., Boston, MA) on an X-ray film according to the manufacturer's protocol.

Example 4

ELISA for the measurement of CD99 in human serum and plasma samples.

For detection of CD99 in human serum or plasma, a sandwich ELISA is developed. For capture and detection of the antigen, aliquots of the anti-CD99 polyclonal antibody (see Example 2) are conjugated with biotin and digoxigenin, respectively.

Streptavidin-coated 96-well microtiter plates are incubated with 100 µl biotinylated anti-CD99 polyclonal antibody for 60 min at 10 µg/ml in 10 mM phosphate, pH 7.4, 1% BSA, 0.9% NaCl and 0.1% Tween 20. After incubation, plates are washed three times with 0.9% NaCl , 0.1% Tween 20. Wells are then incubated for 2 h with either a serial dilution of the recombinant protein (see Example 2) as standard antigen or with diluted plasma samples from patients. After binding of CD99, plates are washed three times with 0.9% NaCl , 0.1% Tween 20. For specific detection of bound CD99, wells are incubated with 100 µl of digoxigenylated anti-CD99 polyclonal antibody for 60 min at 10 µg/ml in 10 mM phosphate, pH 7.4, 1% BSA, 0.9% NaCl and 0.1% Tween 20. Thereafter, plates are washed three times to remove unbound antibody. In a next step, wells are incubated with 20 mU/ml anti-digoxigenin-POD conjugates (Roche Diagnostics GmbH, Mannheim, Germany, Catalog No. 1633716) for 60 min in 10 mM phosphate, pH 7.4, 1% BSA, 0.9% NaCl and 0.1% Tween 20. Plates are subsequently washed three times with

the same buffer. For detection of antigen-antibody complexes, wells are incubated with 100 µl ABTS solution (Roche Diagnostics GmbH, Mannheim, Germany, Catalog No. 11685767) and OD is measured after 30-60 min at 405 nm with an ELISA reader.

Example 5

Statistical analysis of patient data:

Clinical utility of the novel marker CD99 is assessed by measuring its levels in 10 diabetic patients depending on injections of exogenous insulin and comparing the levels with those measured in 10 patients with demonstrated normal beta cell function. . Statistical analysis is performed by standard Student's t-test evaluation with values <0.05 taken as significant.

Claims

1. A method for monitoring the progression of diabetes, comprising the steps of

   a) providing a liquid sample obtained from an individual,
   b) contacting said sample with a specific binding agent for CD99 under conditions appropriate for formation of a complex between said binding agent and CD99, and
   c) correlating the amount of complex formed in (b) to the amount of complex formed in Insulin Resistance.

2. A method for monitoring the efficacy of treatment of diabetes, comprising the steps of

   a) providing a liquid sample obtained from a patient treated against diabetes,
   b) contacting said sample with a specific binding agent for CD99 under conditions appropriate for formation of a complex between said binding agent and CD99, and
   c) correlating the amount of complex formed in (b) to the amount of complex formed in the absence of treatment.

3. A method for the diagnosis of Insulin Resistance comprising the steps of

   a) providing a liquid sample obtained from an individual,
   b) contacting said sample with a specific binding agent for CD99 under conditions appropriate for formation of a complex between said binding agent and CD99, and
   c) correlating the amount of complex formed in (b) to the diagnosis of Insulin Resistance.

4. The methods according to any one of claims 1 to 3, further characterized in that said sample is serum.

5. The method according to any one of claims 1 to 3, further characterized in that said sample is plasma.

6. The method according to any one of claims 1 to 3, further characterized in that said sample is whole blood.

7. Use of protein CD99 as a marker molecule in the diagnosis of Insulin Resistance from a liquid sample obtained from an individual.

8. Use of protein Endoglin as a marker molecule in the early diagnosis of type II diabetes from a liquid sample obtained from an individual.

9. Use according to claim 8, wherein the early diagnosis is made with a sample derived from patients suffering from glucose intolerance.

10. Use of protein CD99 for monitoring the progression of diabetes.

11. Use of protein CD99 for monitoring the efficacy of treatment of diabetes.

12. Use of protein CD99 as a marker molecule for Insulin Resistance in combination with at least one other marker molecule for Insulin Resistance in the diagnosis of Insulin Resistance from a liquid sample obtained from an individual.

13. An immunological kit comprising at least one specific binding agent for CD99 and auxiliary reagents for measurement of CD99.

14. A method of screening for a compound which interacts with CD99, comprising the steps of

a) contacting protein CD99 with a compound or a plurality of compounds under conditions which allow interaction of said compound or a plurality of compounds with CD99; and

b) detecting the interaction between said compound or plurality of compounds with said polypeptide.

15. A method of screening for a compound that may prevent and/or inhibit and/or attenuate Insulin Resistance, comprising the steps of

a) contacting a compound with protein CD99;

b) measuring the activity of protein CD99;

wherein a compound which inhibits the activity of protein CD99 is a compound that may prevent and/or inhibit Insulin Resistance.

16. The method of any one of claims 14 and 15, additionally comprising the step of immobilizing protein CD99 prior to step a) or between steps a) and b).

17. A method of screening for a compound that prevents and/or inhibits and/or delays Insulin Resistance, comprising the step of detecting soluble CD99 secreted from a host in the presence or absence of said compound, wherein a compound that prevents and/or inhibits and/or delays Insulin Resistance is a compound with which the level of CD99 is secreted from a host is changed.

18. Use of protein CD99 as a target and/or marker for screening for a compound that prevents and/or inhibits Insulin Resistance.

19. The methods, uses and kit substantially as hereinbefore described, especially with reference to the foregoing examples.