PHAGOCYTIC ASSAY METHOD

The present invention relates to the field of programmed cell death or apoptosis and in particular to the phenomenon whereby apoptotic cells are rapidly phagocytosed or engulfed by other cells. Specifically, the invention provides assays and materials for use therein, which measure phagocytosis of apoptotic cells. Such assays can be used to identify chemical substances which influence the phagocytic uptake of apoptotic cells and have potential pharmacological activity. The assays of the invention are well adapted for medium-and high-throughput screening using a multi-well plate format.

During development and maintenance of living tissues a large number of cells undergo programmed cell death or apoptosis. This is observed in both vertebrates and invertebrates. For example, it has been shown that in the nematode *C. elegans* 131 cells undergo programmed cell death (Lui and Hengartner (1997) early 1997 International Worm Meeting, Abstract 371). Lysis of the apoptotic cells is potentially harmful since their contents may cause toxic damage to the surrounding tissues. It has been observed that this harmful effect is avoided because apoptotic cells are engulfed and subsequently degraded by other cells. In mammals the engulfing cells may be professional or semi-professional phagocytes such as neutrophils or macrophages or they may be neighbours of the dying cells.

A key feature of the process of programmed cell death, or apoptosis, is the efficiency with which the dying cells are recognized and engulfed by phagocytes (Savill, J.et. al, Immunol Today, 14:131-136, 1993.). Apoptosis triggers a distinct sequence of events

characterized by the expression of phosphatidylserine on the cell surface, DNA fragmentation or laddering and the release of membrane-bound cell fragments called apoptotic blebs and bodies (Cohen, J. J.et. al, Annu Rev Immunol, 10:267-293, 1992.;Kerr J.F.R.et. al, Br J Cancer, 26:239, 1972.). Apoptotic cells and bodies are phagocytosed via various receptors that recognize phosphatitdylserine and other undefined ligands unique to the surface of apoptotic material (Savill, J. S.et. al, J Clin Invest, 83:865-875, 1989.;Fadok, V. A.et. al, J Immunol, 148:2207-2216, 1992.;Savill, J.et. al, Nature, 343:170-173, 1990.). In this way, apoptotic cells, which contain potentially inflammatory factors, are rapidly cleared by neighboring cells acting as semi-professional phagocytes or voracious experts of the macrophage line without inducing an inflammatory response (Fadok, V. A.et. al, J Clin Invest, 101:890-898, 1998.).

The process of apoptosis has been associated with a number of human diseases, including cancer, autoimmune diseases, various neurodegenerative diseases, such as Amyotrophic Lateral Sclerosis, Huntingdon's disease and Alzheimer's disease, stroke, myocardial infarction and AIDS (Thompson, CB, Science 267, pp 1456-1462). Thus, much attention has been focused on elucidating the mechanism of apoptosis and the genes controlling it with a view to developing new therapeutic strategies for these diseases.

Particular diseases have been associated with an impairment of phagocytosis of apoptotic bodies. Examples of such diseases include autoimmune diseases such as systemic lupus erythematosus, (Herrmann, M.et. al, Arthritis Rheum, 41:1241-1250, 1998.), AIDS (Zocchi, M. R.et. al, AIDS, 11:1227-1235, 1997.), acute pulmonary infections (Cox, G.et. al, Am.J

Respir.Cell Mol.Biol., 12:232-237, 1995.) and allergy (Ying, S.et. al, Proc Assoc Am Physicians, 109:42-50, 1997.). It is clear that modulation of phagocytosis of apoptotic cells by drugs is a promising strategy for future therapies.

Phagocytosis of apoptotic cells in vertebrates has been observed to be a very complicated process and how any signal generated by the dying cell is received and transduced by the engulfing cell is not understood.

A swift engulfment of apoptotic cells is observed in the hermaphrodite *C. elegans* and this worm has provided a useful tool for study of the engulfment process. For example, six genes have been identified in *C. elegans* as effecting engulfment known as ced-1, ced-2, ced-5, ced-6, ced-7 and ced-10. Of these ced-6 has been singled out by the present inventors for particular study. It is known that ced-6 maps to chromosome III near daf-4 in *C. elegans* (Lui and Hengartner (1997); Lui and Hengartner (1996) East Coast Worm Meeting Abstract 128). That work showed that two cosmids from this region, F56D2 and F43F12 could rescue *C. elegans* with a ced-6 (n1813) engulfment defect. A 10 kb Xho I subclone from F56D2 with rescuing activity was identified as carrying the ced-6 gene.

The present inventors have identified two human homologues of the *C. elegans* ced-6 (h1ced-6 and h2ced-6), h2ced-6 being a splice variant of h1ced-6 and thought to be a dominant negative version thereof. Both homologues have been shown to be present in the human cell-line THP-1. A surprising degree of sequence homology between hCED-6 and *C. elegans* CED-6 has been found. hCED-6 has a phosphotyrosine binding domain from about amino acid position 11 to about

amino acid position 190 as shown in Figure 4 suggesting its involvement in a tyrosine kinase signal transduction pathway.

h1CED-6 and h2CED-6 proteins and their encoding nucleic acids are useful for carrying out assays as described herein to identify compounds which are inhibitors or enhancers of a signal transduction pathway which promotes phagocytosis of apoptotic cells. In particular they are useful for identifying inhibitors or enhancers of h1CED-6 and h2CED-6 or inhibitors or enhancers of the transcription thereof. Such inhibitors or enhancers may be useful therapeutic agents in the treatment of some of the aforementioned diseases.

In accordance with its first aspect the invention provides an expression vector capable of expressing h1CED-6 or h2CED-6 which vector comprises a sequence of deoxynucleotides encoding the amino acid sequence of Figure 4 or Figure 5 or an amino acid sequence which differs from the amino acid sequence of Figure 4 or Figure 5 only in amino acid changes which are conservative of biological function.

The term "biological function" is defined herein to mean the ability to regulate or affect phagocytosis of apoptotic cells. Amino acid changes which are "conservative" are those which permit biological function to be retained although it may be less than or greater than the level of biological function of the wild-type human CED-6 protein. Such conservative changes may include insertion or deletion of one or more amino acids or substitution of one or more amino acids with another amino acid or acids having similar chemical characteristics. The choice of amino acids for making conservative changes will be well-known to

those skilled in the art.

In a preferred embodiment the expression vector is one comprising the sequence of deoxynucleotides shown from the transcription start codon to the transcription stop codon shown in Figure 2 or Figure 3 and optionally a vector comprising the sequence of deoxynucleotides shown in Figure 2 or Figure 3.

In a particularly preferred embodiment the expression vector of the invention comprises a sequence of nucleotides encoding a reporter gene positioned so that expression of h1CED-6 or h2CED-6 results in expression of the reporter gene. The reporter gene may be positioned 3′ or 5′ to said h1ced-6 or h2ced-6 and may be expressed as a fusion protein with h1CED-6 or h2CED-6. Suitable reporter genes are those which express a fluorescent product such as green fluorescent protein (GFP). Other suitable reporter genes are enzymes, such as β-galactosidase or luciferase, which are capable of acting on a substrate to produce a detectable product, for example a fluorescent product or luminescent product. Examples of expression vectors in accordance with the invention are pGA3103 and pGA3104 which are shown in Figures 29 and 10 respectively.

In another preferred embodiment the expression vector of the invention expresses an epitope tag at the amino and/or carboxy terminal of the h1CED-6 or h2CED-6 protein. An example is the plasmid pBAD/HisA-h1ced-6 the DNA sequence of which is shown in Figure 17.

It will be understood that the expression vectors described above will comprise not only nucleic acid encoding h1CED-6 or h2CED-6 or functional variants thereof but also regulatory sequences operably linked

to said nucleic acid, such as promoter regions that are capable of effecting expression of the DNA fragments. The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner.

Regulatory elements required for expression include promoter sequences to bind RNA polymerase and to direct an appropriate level of transcription initiation and also translation initiation sequences for ribosome binding. For example, a bacterial expression vector may include a promoter such as the lac promoter and for translation initiation the Shine-Dalgarno sequence and the start codon AUG. Similarly, a eukaryotic expression vector may include a heterologous or homologous promoter for RNA polymerase II, a downstream polyadenylation signal, the start codon AUG, and a termination codon for detachment of the ribosome. Such vectors may be obtained commercially or be assembled from the sequences described by methods well known in the art. Promoter sequences which may be used in the expression vectors of the invention include Δ HSP, CMV, SV40, EF-1α, UbC, SG, RSV, TRE/minCMV, HSV TK, 5', LTR and QBISP136 enhanced CMV.

Examples of expression vectors described herein are plasmids but may also be virus or phage vectors. Such vectors will normally possess an origin of replication and one or more selectable markers such as a gene for antibiotic resistance. It is particularly preferred that the expression vectors of the invention are suitable for transfection of mammalian cells and therefore may be provided with a selectable marker accordingly.

In accordance with a second aspect the invention provides a mammalian cell-line transfected with any of the expression vectors described above. Methods of transfecting mammalian cells are well-known to those skilled in the art. The cell-line may be one which is capable of growing in monolayer culture or in suspension culture. Suitable cell-lines are fibroblast cell-lines or epithelial cell-lines such as COS1, BHK21, L929, pc12, CV1, SWISS3T3, HT144, IMR32, HEPG2, MDCK, MCF7, 293, Hela, A549, SW48 or G361. Primary cell-lines such as human dermal FIBs, dermal keratinocytes, leucocytes, monocytes, lyphocytes, dendritic cells or macrophages may also be used. Particularly preferred for use in the phagocytosis assays of the invention are mammalian professional or semi-professional phagocytes of which examples are mouse macrophage cell-line J774 or human monocyte cell-line THP1 which has been shown to express h1CED-6 and h2CED-6 (see Example 3 and Figure 6). Both of the above cell-lines may be referred to as monocyte cell-lines since monocytes are capable of differentiating into macrophages which is the form in which they are used for the assay of the invention.

In accordance with a third aspect the invention provides a method for determining whether a compound is an enhancer or an inhibitor of a signal transduction pathway which promotes phagocytosis of apoptotic cells which method comprises exposing mammalian cells transfected with h1ced-6 or h2ced-6 as described above to apoptotic particles and measuring the rate of phagocytic uptake of said particles by said transfected cells in the presence or absence of said compound. The test compound is preferably added prior to addition of said apoptotic particles.

Suitable apoptotic particles are cells such as

neutrophils, lymphocytes, erythrocytes, lymphocytes or dendritic cells which have been rendered apoptotic and are optionally opsonized by exposure to serum. Cells suitable for forming the apoptotic particles include the cell-lines L929 and PC12. A particularly preferred cell-line for use as an apoptotic particle is the growth factor dependent mouse cell-line Ba/F3. These may be grown in standard culture medium as described in Example 5 and can be rendered apoptotic by growth in the absence of the growth factor IL-3 for a suitable period (for example about 20 hours) prior to use. The apoptotic status of the cells can be determined using, for example, an annexin/propidium iodide labelling kit available from Boeringher Mannheim (Brussels, Belgium). Cells are considered early apoptotic if they are about 20% annexin positive and less than about 5% propidium iodide negative.

The PC 12 cell-line may be rendered apoptotic by growth in standard medium in the absence of nerve growth factor.

As an alternative to the cells described above the apoptotic particles could be a non-living material such as dye-labelled latex beads. 0.1μM, 1μM, 4μM and 10μM beads that have either an amino or carboxylate group are available from Sigma-Aldrich, Bornem, Belgium, or Molecular Probes, Eugene, USA.

In order for the assay described to be suitable for high-throughout compound screening it is preferred that the apoptotic particles bear some kind of detectable label so that it will be readily apparent that the particles have been taken up by the transfected mammalian cell and so that this can be quantified. The inventors have found that this may be easily achieved by stably transfecting the cells

comprising the apoptotic particles with an expression vector comprising a reporter gene. A particularly suitable reporter gene encodes to be β-galactosidase which is capable of cleaving the fluorogenic substrate fluorescein di-b-D-galactopyranoide to a fluorescent compound which may be monitored using standard fluorescence detection equipment. Other fluorescent substrates are available for β-galactosidase. A plasmid pcDNA3.1/HIS/lacz, which expresses β-galactosidase and is suitable for transfecting cells used as apoptotic particles, for example Ba/F3, is shown in Figure 11. Other suitable reporter genes are those encoding fluorescent proteins such as green fluorescent protein or proteins capable of generating a luminescent signal such as luciferase. Plasmids, pEGFP-N3 or PEGFP-C2, available from Clontech, are suitable for transfecting cells used as apoptotic particles with GFP and are shown in Figures 7, 8, 26 and 27. A plasmid "PGL Control" available from Promega is suitable for transfecting cells to be used as apoptotic particles such as Ba/F3 cells with a gene encoding luciferase. The DNA sequence of "PGL control" is shown in Figure 19.

It will be appreciated that the choice of reporter gene for the apoptotic particles may be governed by the presence of any reporter gene in the transfected mammalian cells. For example, the presence of the same reporter gene in the transected mammalian cells transfected with h1 or h2 ced-6 and the apoptotic cells is not *prima facie* desirable because of overlap of signals, although this may not always be the case.

It will further be appreciated that a wide variety of compounds can be tested to see whether they are inhibitors or enhancers of signal transduction pathways which promote phagocytosis of apoptotic

cells. The compound may be of any chemical formula, a polymer or a monomer. For example the test compound may be genomic DNA, cDNA, RNA, PNA, a protein or polypeptide, an amino acid, nucleoside or nucleotide. The compound may be one of known biological or pharmacological activity, a known compound without such activity or a novel molecule such as might be present in a combinatorial library of compounds.

It will be appreciate that where any compound the presence of which results in no or a decreased amount of engulfment of apoptotic particles by the transfected mammalian cells, those cells must be tested for viability. The presence of viable cells will confirm that lack of engulfment is due to the effect of the test compound on phagocytic activity and not just non-specific toxicity.

Furthermore, any compound identified as an inhibitor or an enhancer of phagocytosis of apoptotic cells by the assay described above will be further tested to establish whether the effect is medicated through CED-6. In the case of a compound identified as an enhancer of phagocytosis of apoptotic cells this can be achieved by carrying out a phagocytosis assay exactly as described above with mammalian cells which are not transfected with h1ced-6 or h2ced-6. If the compound is able to induce a phenotype in the untransfected cells which is similar to the phenotype of those cells when transfected with h1ced-6 then it is an indication that the compound in question exerts its effect via CED-6 or via the CED-6 signal transduction pathway.

Similarly, if a compound identified in the above described assay is an inhibitor of phagocytosis of apoptotic cells it can be confirmed whether its effect

is via CED-6 or the CED-6 signal transduction pathway by examining the phenotype of the transfected mammalian cells exposed to the compound. Reversion to a wild-type phenotype is an indication of action via CED-6 or the CED-6 signal transduction pathway.

In a fourth of its aspects the invention provides a method for determining whether a compound is an inhibitor or an enhancer of a signal transduction pathway which promoter phagocytosis of apoptotic cells which method comprises the steps of:

(1) micro-injecting into a mammalian cell a human CED-6 protein comprising the sequence of amino acids shown in Figure 4 or Figure 5 or a sequence of amino ids differing from that shown in Figure 4 or Figure 5 only in amino aid changes conservative of function, and

(2) exposing the mammalian cell produced in step (1) to apoptotic particles and measuring the rate of phagocytic uptake of said particles by said cell in the presence and absence of said compound.

Preferably, the mammalian cell is micro-injected with a fusion protein comprising h1CED-6 or h2CED-6 and a reporter gene which may be any one of the reporter genes described above. Preferred fusion proteins are obtainable by expression from the GFP and h1ced-6 encoding sequences of the plasmids shown in Figures 9 or 28.

All of the preferred features and embodiments described above for assays with transfected mammalian cell-lines can be applied to cells micro-injected with

h1CED-6 or h2CED-6 or fusions thereof as described above.

In a fifth aspect the invention provides a method for determining whether a compound is an inhibitor or an enhancer of a signal transduction pathway which promotes phagocytosis of apoptotic cells which method comprises the steps of:

(1) micro-injecting or transfecting into a mammalian cell a vector expressing RNA antisense to all or a portion of the sequence of nucleotides shown in Figure 2 or Figure 3;

(2) exposing the mammalian cell produced in step (1) to apoptotic particles and measuring the rate of phagocytic uptake of said particles by said cell in the presence or absence of said compound.

Preferably, the antisense DNA comprises a sequence of nucleotides which is capable of hybridizing to a sequence of nucleotides as shown in Figure 2 or Figure 3 under conditions of stringency which are higher than 2xSSC; 0.1%SDS; 25°C to 50°C.

All of the preferred features and embodiments described above for assays with transfected mammalian cell lines can be applied to the cell-lines injected with antisense RNA.

It will be appreciated that the transfected mammalian cells for use in the assays described above may be transfected with h1ced-6 or h2ced-6. Since h2ced-6 is thought to be a dominant negative version of h1ced-6 having an opposite biological effect, transfected

cells can be chosen depending on whether it is desired to identify compounds which are inhibitors or enhancers of apoptotic cell phagocytosis . For example cells transfected with h1ced-6 would be particularly suitable for identifying inhibitors of phagocytosis of apoptotic cells while cells transfected with h2ced-6 would be particularly suitable for identifying enhancers.

It is hereby stated that the invention also relates to any compound identified as an inhibitor or enhancer of a signal transduction pathway which promotes phagocytosis of apoptotic cells as identified in accordance with any of the assay methods described herein.

The nucleotide sequences for h1ced-6 and h2ced-6 are shown in Figures 2 and 3 respectively. In addition cDNAs encoding the alterative splice h2CED-6 and the insert to reconstitute h1ced-6 from h2ced-6 have been deposited at the Belgian Coordinated Collections of Microorganisms (BCCM) at Laboratorium voor Moleculaire biologie - plasmidencollective (LMBP) B 9000, Gent, Belgium in accordance with the Budapest Treaty on 8th June 1998 and hae been accorded the Accession Nos 3868 and 3869 respectively.

Sequences can be obtained in both deposits using T3 or T7 primers (either one or both can be used, they are at different sites of the actual insert). Both are commercially available from Clontech (~1227 and~1228) and sequence is shown below

T7 primer: 5′(TAATACGACTCACTATAGGGAGA)3′

T3 primer: 5′(ATTAACCCTCACTAAAGGGA)3′

In addition to developing assays based on mammalian cells which over or under express human CED-6 protein the present inventors have identified epitopes of h1CED-6 and have generated useful antibodies thereto.

Therefore in a sixth aspect the invention comprises a fragment of human CED-6 protein having the amino acid sequence as shown in Figure 4 wherein said fragment includes the sequence of amino acids HRAFSRKKDKTC, FLGSTEVEQPKGTE or TRNGTQPPPVPSRST. Antibody preparations have been prepared which comprise antibodies to one or more of the above epitopes. Such antibody preparations are obtainable by the method described in Example 6 and their specificity is demonstrated by the Western Blots carried out in Example 7 (see Figures 20 to 25).

The antibodies described above may be used in a method of diagnosing a disease in a patient which is associated with over or under expression of human CED-6 in phagocytic cells. Specifically, there is provided a method for diagnosing a disease associated with the over or under expression of human CED-6 protein in phagocytic cells in an individual which comprises:

(a) obtaining a sample of phagocytes from said individual;

(b) exposing said phagocytes to an antibody preparation as described above;

(c) quantitatively measuring the presence of any immune complexes formed between said antibodies and said CED-6 protein; and

(d) comparing the amount of immune complex

formed with that formed using phagocytes from a control individual.

The antibodies described above may be further used in assays for determining whether a compound is an inhibitor or an enhancer of a signal transduction pathway which promotes phagocytosis of apoptotic cells. Specifically, there is provided a method for determining whether a compound is an inhibitor or an enhancer of a signal transduction pathway which promotes phagocytosis of apoptotic cells which method comprises:

(a) exposing a mammalian cell transfected with an expression vector as described above to the compound to be tested;

(b) exposing said mammalian cell to an antibody preparation as described above;

(c) quantitatively measuring the presence of any immune complex formed between said antibodies and protein expressed by said cells; and

(d) comparing the level of immune complex detected with the amount of immune complex detected in a mammalian cell transfected as described in step (a) which has not been exposed to said compound.

In the above described method the mammalian cell may be selected from COS1, BHK21, L929, CU1, SWISS 3T3, HT144, IMR32, HEPG2, MDCK, MCF7, 293, hela, A549, SW48 or G361 with COS1 cells being particularly preferred. Alternatively, the mammalian cell is a human dermal

FIB, dermal keractinocyte, leucocyte, monocyte, hyphocyte, dendritic cell or macrophage. Preferred are professional phagocytes such as mouse macrophage cell-line J774 or human monocyte cell-line THP-1.

Other uses for the antibodies of the inventions include purification of h1CED-6 and identification of proteins interacting with CED-6 so that the signal transduction pathway can be characterised, detecting over or under expression, cellular localization or post-translational modifications of hCED-6, epitope mapping and identification of active sites and pharmaceutical compositions comprising said antibodies in a suitable carrier or diluent.

In accordance with a seventh aspect the invention also provides a method for diagnosing a disease associated with the over- or under-expression of human CED-6 in phagocytic cells in an individual, which method comprises:

(a) obtaining a sample of phagocytes from said individual,
(b) isolating RNA from said phagocytes,
(c) preparing cDNA from said RNA,
(d) performing a first PCR reaction on said cDNA,
(e) performing a second (nested) PCR on the reaction product of said first PCR reaction,
(f) quantitatively and qualitatively measuring the presence of human ced-6 RNA by analysing the reaction products from the first and second PCR and
(g) comparing the amount and type of reaction products formed in the first and second PCR with that of the reaction products formed using phagocytes from control individuals.

Preferably, the PCR is performed with primers derived from the sequence of h1ced-6 or h2ced-6 as defined herein or derived from the vector used in the generation of cDNA. In particular said first PCR may be performed with primers having nucleotide sequences:

1) cgcaaggatccccatgaaccgtgcttttagcaggaag
2) gatctactaggtactggag

The second PCR may be performed with primers having nucleotide sequences:

1) cgcaaggatccccatgaaccgtgcttttagcaggaag
2) gcggatggtaccgtcgactgctgatacttgagttattctcag

The assay methods described herein, developed by the present inventors for determining whether compounds are enhancers or inhibitors of human CED-6 have been found to be more generally applicable for identifying compounds which influence phagocytosis of apoptotic cells by any mechanism, not necessarily related to human CED-6 or the signal transduction pathway of which it forms a component.

Liu et al. (Liu, Y.et. al, The American Associatian of Immunologists, 1:1999.) describe an assay for identifying compounds which influence phagocytosis of apoptotic cells, in which varying concentrations of the compound to be tested are added to the phagocytes which are subsequently seeded with apoptotic cells. To quantify phagocytosis of apoptotic cells, the authors used a microscopic quantification of phagocytosis in which the uptake of apoptotic cells was shown by electron microscopy and counted by light microscopy with a minimum of cells per slide being counted (Savill, J. S.Wyllie, A. H.Henson, J. E.Walport, M.

J.Henson, P. M.Haslett, C., J Clin Invest, 83:865-875, 1989.).

The presently known techniques for quantitating the phagocytosis of apoptotic cells do not readily lend themselves to high throughput screening of compounds for potential pharmacological activity. This is largely because the known assay techniques rely on microscopic counting of the proportion of phagocytes which have ingested apoptotic cells when exposed to the test compound. However, the present assays overcome this drawback because they can be performed in the multi-well assay format and provide detection systems which do not involve microscopy.

Thus, in accordance with a further aspect of the invention there is provided a method of identifying a compound which is an enhancer or inhibitor of phagocytosis of apoptotic cells which comprises:

a) exposing a mammalian professional or semi-professional phagocyte to an apoptotic mammalian cell which has been stably transfected with a reporter gene capable of generating a signal detectable without microscopy, in the presence of absence of the compound to be tested,

b) removing any apoptotic cells which are not engulfed by said phagocytes and

c) detecting any signal of the reporter gene from said phagocytes;

wherein any difference in signal in the presence of said compound compared to the signal in the absence of said compound is an indication that said compound is an inhibitor or an enhancer of phagocytosis of

apoptotic cells.

Usually, in the above method it is preferable to incubate the phagocytic cells with the test compound prior to addition of the apoptotic cells. A suitable incubation time might be about 15 to 30 minutes.

Suitable phagocytic cells for carrying out the method of the invention are mouse J774 cells or human THP-1 cells as described elsewhere herein. These cells are monocyte cell-lines but are cultured so as to differentiate them into macrophages prior to addition of apoptotic cells.

The apoptotic cells for use in the above method may be apoptotic neutophils, apoptotic lymphocytes or apoptotic erythrocytes. The apoptotic cells may optionally be opsonised by exposure to serum. Preferred apoptotic cells are the adherent cell-lines L929 or PC12 and, in particular, the growth factor dependant mouse cell-line Ba/F3 described elsewhere herein.

The apoptotic cells are stably transfected with a reporter gene of the types and using the methods described above. One particular problem which can arise with the use of reporter genes is that expression of the gene in an apoptotic cell, which is effectively dying, can be much less than in a fully viable cell. If viable cells are present amongst the apoptotic particles added to the phagocytes and there is inadequate washing of the unphagocytosed particles the signal from the viable cells will mask any signal from the apoptotic phagocytosed cells. Athough it is possible to ensure that adequate washing occurs the inventors have developed a particular embodiment where this problem is avoided.

Specifically, this involves using a reporter gene expressing a protein, preferably an enzyme, with a low turnover in the cell such that the living cell and the apoptotic particles have approximately the same protein concentration or enzymatic activity. This overcomes the drawbacks described above. Several possible reporter proteins and substrates have been described in Handbook of fluorescent probes and research chemicals, ed by P. Haugland (Molecular probes, Eugene, OR, USA) which may be used. However, the inventors have found β-galactosidase (lacZ) to be particularly suitable. The enzyme has a relative slow turnover and it is shown that the cells and the apoptotic particles have relatively equal amounts of activity. Furthermore several substrates exist for β-galactosidase (see molecular probes, Eugene, OR, USA) from which the inventors have used FDG mentioned above. This makes it possible to develop a high throughput screen to select for compounds that alter the phagocytosis of apoptotic particles.

The phagocytes for use in the method of the invention may by wild-type cells or they may be transgenic or mutant cells. A mutant cell may have reduced or increased phagocytic activity compared to wild-type. A transgenic cell may be one stably transfected with a gene which when expressed influences the rate of phagocytic activity for apoptotic cells. For example, the mammalian cells may be transfected with h1ced-6 or h2ced-6 as described above, preferably using any of the vectors mentioned herein in the description or drawings.

In another embodiment in accordance with the invention the phagocytes may be transfected with a DNA encoding the cell surface antigen CD36. Expression of CD36 is required for phagocytosis of apoptotic cells by human

macrophages that use either a phosphatidylserine receptor or the vitronectin receptor. (Fadok V.A.et al, J. Immunol 1998 Dec 1.161(11):6250-7.) Transfection may be carried out by any of the methods described herein and preferably using a vector comprising a DNA sequence encoding CD36 as shown in Figure 31 or the entire vector of Figure 31.

The methods of the invention are all performed in a multi-well plate format and are therefore particularly suitable for mid-to-high throughput screening. In a preferred embodiment, the multi-well plates have 96 wells, but the invention is also applicable to multi-well plates with another number of wells, which include but is not restricted to plates with 6, 12, 24, 384, 864, 1536 wells.

All of the methods of the invention require the detection of a signal which quantitates the phagocytosis of apoptotic cells in the presence of the compound under test. It is an essential feature of the methods of this invention that this signal (also referred to as the read-out) is detected using a non-visual detection means. As used herein the term 'non-visual detection means' refers to any means of detecting a signal which does not require visual inspection of the human eye including inspection through a microscope. The use of a non-visual detection system represents a major advantage over previously known screening methods which require visual inspection of the cells by eye in order to detect uptake of apoptotic cells by phagocytes.

To allow for the non-visual detection of the apoptotic cells, in the high to mid-throughput screening in the phagocytosis assay, the reporter gene must be capable of generating a signal which is detectable by an

automatic plate reader, such as the victor2 (Wallac, Turku, Finland). An automatic plate reader which detects a fluorescent signal is most preferred.

By generating a signal which can be read by an automatic multiwell plate reader quantitatve measurements can be made and this allows for the assessment of the effect of many compounds at once, as well as comparison of the effects between the compounds.

It is further pointed out that the compounds to be tested may be any of the types of compounds described above and that where a particular compound results in a reduced signal or no signal for the phagocytes, the phagocytes will be tested for viability to rule out non-specific toxicity of the compound in question.

In the non-limiting examples which follow reference is made to the following Figures:

FIGURE 1 shows the construction of 2416bp consensus sequence which was obtained from EST, RACE and colony hybridization (see Example 1). The sequence was compiled by using a a159394 as template and primers as indicated in multiple alignment. rcc stands for reverse complement. Both ced-6 and hced-6 are indicated above the multiple alignment; pGA101 was picked up by colony hybridization;

FIGURE 2 shows the consensus DNA sequence of h1ced-6 (2416 bp). Start and stop codons are in bold and underlined. Alternatively, spliced region is underlined;

FIGURE 3 shows the alternatively spliced DNA sequence of h2ced-6. Start and stop condon in bold and

underlined;

FIGURE 4 shows the amino acid sequence of hCED-6. Number of residues: 304, Molecular weight 34.4kDa. again the alternatively spliced region is underlined;

FIGURE 5 shows the amino acid sequence of h2CED-6, the alternatively spliced version;

FIGURE 6 shows gel analysis of the nested PCR products generated as described in Example 3; the lanes are loaded as follows: (1) 100bp marker, (2) primary living neutrophils, (3) primary apoptotic neutrophils, (4) primary macrophages, (5) primary macrophages interacted with apoptotic neutrophils, (6) J774, (7) COS-1, (8)THP-1;

FIGURE 7 shows the DNA sequence of the commercially available Clonetech vector pEGFP-N3 comprising the reporter gene GFP as used in Examples 4 and 8;

FIGURE 8 shows a plasmid map of pEGFP-N3;

FIGURE 9 shows the DNA sequence of plasmid pGA3104, as used in Examples 4 and 8, which comprises h1ced-6 in the multicloning site of pEGFP-N3;

FIGURE 10 shows a plasmid map of pGA3104;

FIGURE 11 shows a DNA sequence of commercially available plasmid pcDNA3.1/His/LacZ used for stable transfection of Ba/F3 cells (see Example 4);

FIGURE 12 shows fluoresence intensity as a function of transfected cell concentration when β-galactosidase is reacted with the fluorogenic substrate fluorescein di-b-D-galactopyranoside (FDG);

FIGURE 13 shows the effect of (FDG) concentration on the read-out of the assay of Example 5;

FIGURE 14 shows the effect of incubation time on the read-out of the assay of Example 5;

FIGURE 15 shows the effect of serum concentration in medium of Ba/F3 cells on the assay of Example 5;

FIGURE 16 shows the location of the epitopes in h1CED-6 used for generating polyclonal antibodies;

FIGURE 17 shows the DNA sequence of the plasmid pGA1028 (pBAD/His A/-hced-6) used in Example 7;

FIGURE 18 shows a plasmid map of pGA 1028;

FIGURE 19 shows the DNA sequence of commercially available Promega plasmid pGL2 which is suitable for introduction of reporter gene luciferase into Ba/K3 cells;

FIGURES 20 to 25 show the results of the immunoblots carried out in Example 7. In all these figures the lanes are loaded as follows:

Lane 1: Prestained SDS - PAGE Standards Low Range (Bio Rad - Hercules, CA, USA),

Lane 2: pBAD/His A (Invitrogen, Leek, The Netherlands)

Lane 3: pGA1028 (pBAD/HisA/-h1CED-6)

FIGURE 20 shows gel stained with antibodies to the epitope EP 990044 as identified in Example 7 and control antibodies Anti-Xpress Ab (Invitrogen, Leek,

The Netherlands) and Mouse 1g, horseradish peroxidase-linked whole antibody (from sheep) (ECL Western blotting detection reagents and analysis system, Amersham Pharmacia Biotech, UK, England);

FIGURE 21 shows gel stained with antibodies to epitope 990044 and immune serum as described in Example 7;

FIGURE 22 shows gel stained with antibodies to epitope 990045 as identified in Example 7 and control antibodies as described for figure 20;

FIGURE 23 shows gel stained with antibodies to epitope 990045 and immune serum as described in Example 7;

FIGURE 24 shows gel stained with antibodies to epitope 990046 and with control antibodies as described for Figure 20;

FIGURE 25 shows gel stained with antibodies to epitope 990046 and with immune serum as described in Example 7;

FIGURE 26 shows the DNA sequence of the commercially available Clonetech vector pEGFP-C2 comprising the reporter gene GFP as used in Example 8;

FIGURE 27 shows a plasmid map of pEGFP-C2;

FIGURE 28 shows the DNA sequence of plasmid pGA3103 as used in Example 8 which comprises h1ced-6 in the multicloning site of pEGFP-C2;

FIGURE 29 shows a plasmid map of pGA3103;

FIGURE 30 shows Western blot of cell lysates from COS-1 cells transfected with MOCK (negative control for

transfection), pEGFP-N3, pGA3103 and pGA3104; control lysates for actual co-immunoprecipitation from Ba/F3 cells incubated with or without the first antibody; positive control lysates from EGF-stimulated A431 cell lysates for anti-phosphotyrosine antibody. Blot A was probed with a mouse monoclonal 1gG2 which detects tyrosine-phosphorylated proteins in cell lysates; blot B was probed with polyclonal antibody which reacts with green fluorescent protein; and blot C was probed with rabbit antiserum to h1CED-6. MW of h1CED-6 is 34435.39; Mw of GFP is 26886.32; and Mw of the fusion protein GFP-CED-6 or CED-6-GFP is 62385.95;

FIGURE 31 shows the DNA sequence of plasmid pGA1058 which comprises a DNA sequence encoding the cell surface receptor CD36 inserted in the multicloning site of pEGFP-N3;

FIGURE 32 shows a plasmid map of pGA1058;

FIGURE 33 shows the percentage annexin and propidium iodide positive cells in a cell population of Ba/F3 cells as a function of time after withdrawal of IL-3;

FIGURE 34 shows the effect of temperature on FDG incubation, live and apoptotic Ba/F3 cells were added to macrophage cell-line J774. After the phagocytosis assay, FDG (10µM) was incubated for 1h at 4°C, 20°C and for 10 and 20 min at 37°C.

EXAMPLE 1

Extensive searches (tblastn) with the ced-6 sequence (Figure 2 Consensus DNA Sequence of hced-6) against the public domain databases (EST, Genbank, EMBL, Swissprot and PIR) revealed statistically significant homologies to some ESTS at the carboxyterminal region

of the protein (AA443368, AA431995, R33389, R53881). One Est (T48513) showed homology to the carboxyterminal of the PTB domain and the beginning of the charged region. For 5' RACE analysis a Marathon-ready cDNA colorectal adenocarcinoma, library was used from Clontech. The position of the primers used for RACE and sequencing is indicated in Figure 1. By subsequent cloning and sequence analysis additional sequence information was obtained. Using this additional sequence information and subsequent rounds of database searching (blastn) revealed additional EST, which enabled us to construct a consensus of approx. 2400 bp. This sequence was further extended and verified by colony hybridization and sequencing additional RACE products.

EXAMPLE 2

RNA Blots:

A human multiple tissue Northern (MTN-1, Clontech) containing in each lane 2 mg of poly A + RNA from eight different human tissues (heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas) and a MTN-II human multiple tissue Northern, containing in each lane 2 mg of poly A + RNA from spleen, thymus, prostate, testis, ovary, small intestine, colon and peripheral leukocyte, were hybridized according to the manufacturer's instructions and washed out in 0.1 x SSC, 0.2% SDS at 55°C. Also from Clontech, a poly A + RNA blot from human cancer cell lines (melanoma G361, lung carcinoma A549, colorectal adenocarcinoma SW480, Burkitt's lymphoma Raji, lymphoblastic Leukemia Molt-4, chronic myelogenous leukemia K562, Hela S3 and promyelocytic leukemia HL60) was tested.

Expression pattern of hCED-6 in normal human tissues

and cancer cell lines by Northern blotting is shown in Table I below:

A) Human Multiple Tissue Northern (MTN) Blot B) Human Multiple Tissue Northern (MTN) Blot II C) Human Cancer Cell Line Multiple Tissue Northern (MTN™) Blot.

TABLE I

A)

	heart	brain	placenta	lung	liver	skeletal muscle	kidney	pancreas
Expression level	+		+++	+		++	+	+
length (kb)	3,6		3,6	3,6		3,9	3,6	3,6

B)

	spleen	thymus	prostate	testis	ovary	small intestine	colon (mucosal lining)	peripheral blood leukocyte
Expression level	+		+	++	+	+	+	
length (kb)	3,6		3,6	3,9	3,6	3,6	3,6	

C)

	promyelocytic leukemia HL-60	HeLa cell S3	chronic myelogenous leukemia K-562	lymphoblastic leukemia MOLT-4	Burkitt's lymphoma Raji	colorectal adeno-carcinoma SW480	lung carcinoma A549	melanoma G361
Expression level		++	+++			+++	+++	+
length (kb)		3,6	3,6			3,6	3,6	3,6

EXAMPLE 3

Detection of the CED-6 (h1CED-6) and its splice variant (h2CED-6) in phagocytic cell lines.

Cell line THP-1 (ATCC no: TIB-202), a human monocyte celline that can be differentiated into a macrophage cell with PMA (Sigma-Aldrich, St-Louis, MO, USA), was cultivated under standard conditions in RPMI 160 medium containing 2mM L-glutamine, 1.5g/L sodium bicarbonate, 4.5 g/L glucose, 10mM HEPES, 1 mM Sodium pyruvate, 0.05mM β-mercaptoethanol. (all purchased from gibcoBRL, Life Technologies, Merelbeke, Belgium)

RNA has been isolated from this cell line using the RNeasy mini kit from qiagen (Westburg, Leusden, the Netherlands), according the instructions of the manufacture, or with minor modifications thereof.

Starting from this RNA, first strand cDNA was generated using the Ready-To-Go T-primed First-strand kit from Pharmacia Biotech (Piscataway, NJ, USA), according the instructions of the manufacture, or with minor modifications thereof.

The generated cDNA was used in a PCR protocol to generate DNA fragments using primers:
oGA131: 5'-CGCAAGGATCCCCATGAACCGTGCTTTTAGCAGGAAG-3'
445-10934-13R: 5'-GATCTACTAGGTACTGGAG-3'
PCR was performed with the TaKaRa ex Taq kit (Takara Shuzo CO., LTD, Shiga, Japan) according the instructions of the manufacture, or with minor modifications, Plasmid pGA1025, harboring the h1ced-6 gene was used as positive control.

In summary:

PCR on the first-strand cDNA isolated from the Ready-To-Go T-primed First-strand kit contained the entire cDNA as made, 0.4 µl oGA131 (100pmol/µl), 0.4 µl 445-10934-13R (100pmol/µl),0.5 µl exTaq 5U/µl, 65.7 µl water.

PCR on the positive control contained, 10 µl buffer exTaq 10x, 10 µl dNTP mix exTaq 10x, 0.4 µl oGA131 (100pmol/µl), 0.4 µl 445-10934-13R (100pmol/µl), 2 µl pGA1025, 76.2 µl water.

PCR-program:

95°C	1′	
45°C	30″	40x
72°C	30″	
72°C	7′	
4°C	∞	

2 µl of each PCR reaction and 1 µl from the positive control PCR reaction was used to perform a nested PCR, with following primers:

oGA131: see above

oGA141:5′-GCGGATGGTACCGTCGACTGCTGATACTTGAGTTATT CTCAG-3′

PCR was performed with the TaKaRa ex Taq kit (Takara Shuzo CO., LTD, Shiga, Japan) according the instructions of the manufacture, or with minor modifications.

The mastermix: 5 µl buffer exTaq 10x, 5 µl dNTP mix exTaq 10x, 0.2 µl oGA131 (100pmol/µl), 0.2 µl oGA141 (100pmol/µl), 0.5 µl exTaq 5U/µl, 37.1 µl water.

Program:

94°C	4′	
94°C	1′	
45°C	30″	35x
72°C	30″	
72°C	7′	
4°C	∞	

10 μl nested-PCR product was analyzed on gel using standard protocols (Molecular Cloning, a laboratory manual, Sambrook et al, 1989, CSHL press).

The above procedure was repeated for primary living neutrophils, primary apoptotic neutrophils, primary macrophages, primary macrophages interacted with apoptotic neutrophils, mouse monocyte cell-line J774 and COS-1 cells. The results are shown in Figure 6. Remarkably, only in cell-line THP-1 could h1ced-6 and its splice variant h2ced-6 be detected (see lane 8 of Figure 6).

EXAMPLE 4

Stable cell lines of human CED-6

J774 murine monocyte tumour cell line (Morland and Kaplan, 1978, Exp. Cell Res. 115:53-61; Morland and Kaplan, 1978, Exp. Cell Res. 115:63-72 Accession No ATCC TIB67 - also described as J774A.1)cultivated in DMEM, with glutamaxI, 10% myoclone serum (all from GIBCOBRL, Life Technologies, Merelbeke, Belgium), were transfected by electroporation, with the plasmids pEGFP-n3 (Clonetech, Palo Alto, CA, USA)(Figures 7 and 8), mock transfection,
pGA3104, h1CED-6/GFP fusion (Figures 9 and 10)
Salmon Sperm DNA; negative control.

Electroporation was performed with Easyject Plus electroporator system from Equibio Ltd (Immunosource, Halle-Zoersel, Belgium), using following protocol:
3 x 10^6 cells were placed in 800μl cell culture medium, and 30 μg DNA was added
The settings of the Easyject Plus electroporator were: double pulse:
Voltage I = 750V, Capacitor I = 25 μF, Resistance I = 99R, Interpulse delay= 0

Voltage II 150 V, Capacitor II = 1500 μF, Resistance II = 99R, Optipulse option
3200 μl of electroporated cells per construct were seeded into a 175 cm^2 culture flask, and selected with G418 antibiotic (400 μg/ml) (Duchefa, Haarlem, The Netherlands) after 72h. Subclones of clones were obtained and checked for GFP expression.

EXAMPLE 5

Phagocytosis assay

Preparing the phagocytes

Monocyte cell line J774 stably transfected with pEGFPn3 or pGA3104, or pGA1058 were plated at a density of 1 x 10^5 in a black 96-well plate for 36h at 37 °C and 5% CO_2 in advance of performing the phagocytosis assay.

Preparing the apoptotic cells

Growth factor dependent cell line Ba/F3 stably transfected with β-galactosidase as reporter gene is used as source of apoptotic cells. A suitable plasmid for transfecting Ba/F3 is pcDNA3.1/His/LacZ as shown in Figure 11. Ba/F3 cells which are IL-3 dependent mouse clones (Palacios and Steinmetz, 1985, Cell 41:727-734; Palacios et al, 1984, Nature 309:126-131), were grown in DMEM with glutamaxI, 10% FCS, 1% antibiotics (all from GIBCOBRL, ibid.), and 10% supernatant from WEHI-3 culture.
WEHI-3 (ATCC no.: TIB-68) produces IL-3 when grown in culture medium: RPMI 1640 with glutamaxI, 10% FCS, 3.6μl β-mercaptoethanol per 1 litre.
Ba/F3 cells were split ½ two days before the interaction assay (exponential growth phase) and Ba/F3 cells (5 x 10^6/ml) were cultured without growth factor IL-3 for 20h in advance of performing the assay.
Apoptotic Ba/F3 cells were monitored by the

annexin/propidium iodide labeling Kit from Boeringher-Mannheim (Brussels, Belgium). Ba/F3 cells are early apoptotic if 20% annexin positive and less than 5% propidium iodide negative. Ba/F3 cells cultured with growth factor IL-3 were used as a negative control. Results of the annexin/propidium iodide test are shown in Figure 33.

Adding the apoptotic cells to the phagocytes

100 μl Ba/F3 cells (1 x 10^7 cells/ml) were added to wells containing stably transfected J774 and to the negative control wells and incubated at 37°C for periods ranging from 20 minutes to 5 hours after which the apoptoptic cells were removed from all wells. Phagocytes were washed three times in PBS buffer (GIBCOBRL, ibid.), being careful not to dislodge any of the cells.

Read-out

Phagocytosis by the J774 cells of the apoptotic bodies was measured by detecting the β-galactosidase as expressed in the Ba/F3 cells. Detection was performed with a fluorogenic substrate, Fluorescein di-b-D-galactopyranoside (FDG)(Molecular probes, Eugene, OR, USA). 10 μM FDG was added to the wells and incubated for 1h at room temperature in the dark. FDG is sequentially hydrolysed to FMG and fluorescein by the activity of the β-galactosidase, and the green fluorescein emission was measured in a standard plate reader using 480mm excitation, 520mm emission and the appropriate sensitivity settings.
For calibration purposes fluorescent read-out was determined for different Ba/F3 concentrations. The fluorescent read-out as a function of cell concentration is shown in Figure 12.
Further experiments were carried out varying the concentration of FDG, the incubation time of the

assay, the temperature of the assay and the concentration of serum in the Ba/F3 medium. The results are shown in Figures 13, 14, 15 and 34 respectively.

Compound Screening

The above described phagocytosis assay is used to screen compounds for their ability to influence the level of phagocytosis of apoptotic cells by professional phagocytes. The test compound or compounds can be added to the test wells approximately 30 minutes before addition of the Ba/F3 cells to the wells. Because of the multiwell format of the assay and automatic readout of fluorescence using standard equipment the assay is ideally suited to high through-put compound screening.

It will be understood that where any compound the presence of which results in no fluorescence or reduced fluorescence compared to phagocytic cells not exposed to the compound, the cells in that well will be tested for viability using commercially available reagents such as the Live/Dead Viability/Cytotoxicity Kit from Molecular Probes(Eugene, USA). This kit provides a two-colour fluorescence cell viability assay that is based on the simultaneous determination of live and dead cells with two probes, calcein AM and ethidium homodimer, that measure two recognised parameters of cell viability, intracellular esterase activity and plasma membrane integrity, respectively. This kit is suitable for use with fluoresence multiwell plate scanners. The presence of viable cells will confirm that the lack of fluorescence is due to the effect of the compound on phagocytic activity and not just non-specific toxicity.

Furthermore any compound identified as an inhibitor or an enhancer of phagocytosis of apoptotic cells by the assay described above will be further tested to confirm the effect is medicated through CED-6. In the case of a compound identified as an enhancer of phagocytosis of apoptotic cells this can be achieved by carrying out a phagocytosis assay exactly as described above with J774 cells which are not transfected with hlced-6 or h2ced-6. If the compound is able to induce a phenotype in the J774 cells which is similar to the phenotype of those cells when transfected with hced-6 then it is an indication that the compound in question exerts its effect via CED-6 or via the CED-6 signal transduction pathway.

Similarly, if a compound identified in the above-described assay is an inhibitor of phagocytosis of apoptotic cells it can be confirmed whether its effect is via CED-6 or the CED-6 signal transduction pathway by examining the phenotype of the transfected J774 cells exposed to the compound. Reversion to a wild-type phenotype is an indication of action via CED-6 signal transduction pathway.

EXAMPLE 6

Polyclonal antibodies directed to human CED-6

Polyclonal antibodies where raised in rabbits against the following ced-6 epitopes:

EP990044 H2N - NRA FSR KKD KTC CONH2

EP990045 H2N - CFL GST EVE QPK GTE CONH2

EP990046 H2N - CTR NGT QPP PVP SRS T CONH2

Location of these epitopes in the ced-6 protein is shown in Figure 16.

The polyclonals were raised by Eurogentec Bel, Herstal, Belgium, using following protocol:

Day 0 : taking of pre-immune serum followed by the

first immunisation
Day 14 : second immunisation
Day 28 : third immunisation
Day 38 : blood sampling (shipping 2ml)
Day 56 : fourth immunisation
Day 66 : blood sampling (shipping 2ml + 20ml)
Day 80 : complete bleeding.

EXAMPLE 7

Testing Antibodies by Western Blot

Transformation of plasmid pGA1028(pBAD/His A-hCED-6) (see Figures 17 and 18) was done in TOP 10 competent cells. Furthermore pBAD/His was transformed in the same E. coli cells as a negative control. pBAS/HisA and E. coli TOP 10 were purchased from Invitrogen (Leek, The Netherlands).

The pBAD-vectors expression system was used, as it is known to be an efficient expression system. In the presence of arabinose, expression from pBAD is turned on while the absence of arabinose produces very low levels of transcription from pBAD. A pilot expression was carried out according the instructions of the manufacturer, in which the amount of arabinose was varied to determine the approximate amount of arabinose needed for maximum expression of your protein. The protocol according to the manufacturer (invitrogen, Leek, The Netherlands) was used. Expression was scaled up using the same protocol.

Purification of protein was performed from the E. coli cells transformed with pGA1028: 5 ml lysis buffer (10 ml TE 1x pH 8, 0.5 mg/ml lysozyme, 0.1mg/ml DNAse, 100 μl 1M $CaCl_2$ 400 μl protease inhibitor 25x) was added to the pellet of a 50ml expression induced culture, and the pellet was resuspended in this lysis buffer.

The suspension was placed for 30 mins on ice and sonicated 3 times for 5" (high density), after which the suspension was treated with 3 cycles of freeze-defreeze (liquid nitrogen - 42°C), and placed for 30 min at 37°C. The suspension was centrifuged for 5' at maximal speed. The pellet which contains the insoluble fraction and also the hCED-6 fusion protein was resuspended in 1 ml 2M urea and shaken for 5' at 1200 rpm. This suspension was centrifuged for 5' at maximal speed, and the supernatant was used for gel electrophoresis and Western blotting. 25 µl supertant and 25 µl premixed Laemmli Sample Buffer (Bio Rad-Hercules, CA, USA) was mixed.

Proteins from the negative control were not purified. E. coli transformed with pBAD/His were prepared by pelleting 1 ml of induced E. coli culture, and resuspending the pellet in 1 ml of premixed Laemmli Sample Buffer (Bio Rad- Hercules, CA, USA). As such this suspension can be used for PAGE Gel electrophoresis.

Preparation of samples to load on a gel:
Both the samples were boiled for 5' and placed on ice prior to loading. 25 µl samples were loaded on a Ready Gel, 50 µl well TrisHCl, 4-15% (Bio Rad-Hercules, CA, USA) and electrophoresis was performed according to the manufacturer's instructions. The proteins of the gel were transferred on nitrocellulose membrane (Trans-blot Transfer medium, Bio Rad-Hercules, CA, USA) with a MiniTransBlot electrophoresis cell (Bio Rad- Hercules, CA, USA) according to the instructions of the manufacturer (Bio Rad- Hercules, CA, USA).

Western Blot was performed according to the providers of the antibodies and the detection kit.

A first antibody, in the western blots, immune serum or pre-immune serum of the rabbits was used in a dilution of 1/2000 in PBST (1.44 g/L KH2PO4, 90 g/L NaCl, 7.75 g/L Na2HPO4.7H20, 0.1% Tween)
A second antibody: anti-rabbit, horseradish peroxidase-linked whole antibody (from donkey) diluted 1/4000 in PBST (0.1%) (ECL Western blotting detection reagents and analysis system, amersham pharmacia biotech, UK, England).
All incubations were performed according to the manufacturer, with the following modifications. The first antibody was incubated overnight in PBST, instead of TBST. Detection of the antibodies was done according to the manufacturer's instructions (ECL Western blotting detection reagents and analysis system, Amersham Pharmacia Biotech, UK, England). Stripping and reprobing membranes after ECL detection kit, was done according to the manufacturer's instructions (ECL Western blotting detection reagents and analysis system, Amersham Pharmacia Biotech, UK, England).

Western Blot with control antibodies was done according the manufacturer's instructions of the anti-HisA antibodies (invitrogen, Leek, The Netherlands), The antibody, designated Anti-Xpress antibody diluted 1/5000 in PBST (0.1%) (invitrogen, Leek, The Netherlands) was used as first antibody.
Second antibody: Mouse Ig, horseradish peroxidase-linked whole antibody (from sheep) diluted 1/4000 in PBST (0.1%) (ECL Western blotting detection reagents and analysis system, Amersham Pharmacia Biotech, UK, England).
The staining of the antibodies in both experiments were performed according to the instructions of the manufacturer (ECL Western blotting detection reagents and analysis system, Amersham Pharmacia Biotech, UK,

England).

The results are shown in Figures 20 to 25.

EXAMPLE 8

Interaction of H-CED-6 With Phospharylated Tyrosine Proteins

Co-immunoprecipitation

The antibodies raised against the three epitopes of h1CED-6 were used in western blotting to detect CED-6 interactions with phosphorylated tyrosine proteins and to identify CED-6 interacting proteins.

Transfection

Transfection of COS-1 cells was performed with plasmids pGA3103 (see Figures 26 to 29) and pGA3104 (see Figures 7 to 10) in a 175 cm^2 flask (1 x 10^7 cells). As a negative control: MOCK and pEGFP-N3 were used. Full length human ced-6 (in frame with GFP, both N and C terminal fusions, internal control) were investigated. COS-1 cells were transfected with lipofectamine Plus reagent (GIBCO-BRL). The protocol from Life Technologies was followed and the volumes that were used are shown in Table 2.

MOCK transfected cells are a negative control for transfection. In place of adding DNA, the solvent of the DNA only is added to the cells. Solvent of DNA is TE buffer, pH=8: 1M Tris (ICN) Ph=8 and 0.5 M EDTA (Merck-Belgolabo) pH=8 in H20.

TABLE 2

. Lipofectamine transfection (Life Technologies) of COS-1 cells in a 175 cm^2 flask

Culture flask	Construct	Conc. DNA	DNA	DNA	Optimem	Plus reagent	Optimem	Lipo fecta mine	Optimem	Optimem
		µg/µl	µg	µl	µl	µl	µl	µl	µl	ml
175 cm^2	MOCK	-	-	12 (TE)	1125	60	1125	90	2250	15
175 cm^2	pEGFP-N3	1	12	12	1125	60	1125	90	2250	15
175 cm^2	PGA3103	1	12	12	1125	60	1125	90	2250	15
175 cm^2	PGA3104	1	12	12	1125	60	1125	90	2250	15

As a positive control for phosphorylation, the β-chain of the IL-3 receptor of Ba/F3 cells which is phosphorylated was used.

COS-1 cell lysates were prepared using DIGITONIN (as gently as possible, not to disturb the interaction) and phosphatase inhibitors added (protease inhibitors, preferably cocktailpils or pefablock) to the lysis buffer.

- Low stringency DIGITONIN based buffer:
  Buffer with DIGITONIN in 10 ml bidi
  - 1 % digitonin (Serva 19551, MW 1229.3) SERVA 2% stock= 250 mg in 12.5 ml
  - 10 mM triethanolamine pH 7.8 (Sigma-Aldrich; Bornem, Belgium10X stock 100 mM= 185,7 mg in 10ml pH 7.8 (5ml in 50ml)
  - 0.15 M NaCl (MW 58.44) 87.66 mg per 10ml
  - 2 mM Na_3VO_4 (Sigma-Alrich, Bornem , Belgium) 3.687 mg per 10 ml
  - 2 mM EDTA (Titriplex III; MW 372.24) (Darmstadt, Germany)
    7.444 mg per 10 ml
  - 200 U/ml aprotinin - Trazilol (Sigma-Aldrich, Bornem, Belgium)
    1 mg = 11 TIU = 9900 KU
    200X stock: 10 mg in 2 ml PBS = 49500 KU (50μl in 10ml)
  - 1 mM Pefabloc (Merck , Darmstadt, Germany) 2.4 mg per 10 ml

Lysis of cells

- Transfected cells were washed 2 x in PBS Dulbecco's (GIBCOBRL) in falcon
- Cells were scraped and pellet resuspended in 300μl lysis buffer.

- All manipulations were carried out at 4°C.
- The preparation was centrifuged at 4000 rpm and the supernatant transferred to a new tube.

Preclearance

- Protein G sepharose CL-4B beads (Amersham Pharmacia, Roosendaal, the Netherlands) were supplied freeze dried in the presence of additives. These additives were washed away at neutral pH and ethanol replaced with lysis buffer.
- 50% v/v Protein G sepharose suspension:

  1 ml 50/50 v/v Protein G sepharose was pipetted and centrifuged at high speed for 5 sec. It was then aspirated and resuspended in equal volume of lysis buffer. Washing was repeated three times.
- To 300 µl of lysate was added 50 µl of protein G sepharose CL-4B beads (Amersham Pharmacia, ibid.) and this was reacted for 1 hour.
- It was then centrifuged 10 sec at 14 000 rpm and 4°C and the supernatant transferred to a new tube.

Co-immunoprecipitation

- COS-1 lysates: 5 µl anti-green fluorescent protein (GFP) polyclonal antibody rabbit (Immunosource, Halle-Zoersel, Belgium) was added.
- Lyophilized form was dissolved in 100 µl distilled water then frozen at -20°C
- Ba/F3 lysate number 5: 5 µl of rat antibody to β-chain of IL-3 receptor (Van der Heyden J., Devos R., Plaetinck G., Fache I., Fiers W., Tavernier J. 1991. Characterization of the murine IL-5 receptor complex with the use of a panel of monoclonal antibodies. Relationship to the murine IL-3 receptor. J Immunol. 147:3413-3418) was added.

- Ba/F3 lysate number 6: no antibody was added.
- Samples were incubated between 4 h and 24 h (overnight) at 4 °C, rotating
- 50 µl protein A beads were added and incubated for 1 hour at 4°C.
- Samples were centrifuged for 3 min at 3000 rpm (4°C)
- Beads were resuspended in 800 µl lysis buffer, inverted several times or rotated for a few minutes and centrifuged at 3000 rpm for 3 minutes (4°C). This was repeated three times and on the last occasion the wash buffer was removed with a capillary tip.
- Beads were suspended in 20 µl SDS loading buffer (with -mercapto)
- **Lysate number 7= EGF-stimulated A431 Cell lysate (positive control for anti-phosphotyrosine)** (Upstate Biotechnology, cat. No. 12-302)
- 2.5µl of β-mercaptoethanol was added to 100µl of lysate and samples boiled.
- Samples were centrifuged for 3 min at 3000 rpm (4 °C) and SN was loaded on SDS PAGE using prestained SDS-Page standards low range from BioRad (cat.no 161-0305)

Western-blotting

- Cell lysates were transferred to nitrocellulose with:
- Transfer buffer= 48 mM Tris, 39 mM glycine, 20% methanol, pH 9.2 (5.82 g Tris, 2.93 g glycine in H_2O, 200 ml methanol, to 1 L H_2O
- Blocking buffer: 1x PBS, 0.1% Tween, 5% milk powder, incubate blot overnight
- Gel was probed with anti-phosphotyrosine (cat. 05-321, Upstate Biotechnology): 1 µg/ml for 3h and

washed twice with PBS, 0.1% Tween for 5 min

- Proteins were visualized using 1:4000 goat anti-mouse horseradish peroxidase (cat.no RPN 2108, Amersham pharmacia biotech) as second Ab for 1h at RT.
- Blots were washed twice with PBS, 0.1% Tween for 5 min, twice with blocking buffer for 5 min and twice with H_2O (5min)

ECL Western blotting analysis system

ECL Western blotting detection reagents from Amersham Pharmacia Biotech (cat.no RPN 2108) were used.

Stripping and reprobing blot after ECL detection kit

The membranes were stripped of bound antibodies and reprobed. Membranes were stored wet in saran wrap at 4°C after each immunodetection.

- The membrane was submerged in stripping buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7) and incubated at 50 °C for 30 min with occasional agitation.
- The membrane was washed for 2 x 10 min in PBS, 0.1% Tween at room temperature using large volumes of wash buffer.
- The membrane was blocked by immersing in blocking buffer for 1h at RT.
- Immunodetection was performed with anti-green fluorescent GFP and repeated with anti-human CED-6

Results

Western blots of all cell lysates probed either with anti-phosphotyrosine, anti-green fluorescent protein (GFP) or with rabbit sera against CED-6 are shown in

Figure 30, blots (A), (B) and (C). One band between 49 and 74K stained with anti-phospotyrosine is present in the COS-1 cell lysates transfected with fusion proteins of GFP and CED-6 and is not present in the control COS-1 cell lysates. By probing the western blot with anti-GFP and anti-CED-6 the same band between 49 and 74K was stained.

Conclusion

Fusion proteins CED-6-GFP and GFP-CED-6 are both tyrosine phosphorylated. Their molecular weight is 62385.95K which represents the band between 49 and 74K that is stained positive for anti-phosphotyrosine, anti-green fluorescent protein (GFP) and anti-CED-6.

EXAMPLE 9

Stable cell lines transfected with human cell surface receptor CD36.

J774 murine monocyte tumour cell line was transfected by electroporation with plasmid pGA1058 shown in Figure 31 and 32. The methods used were as described in example 4 for human ced-6.
The transfected cell-line was used as a positive control in carrying out phagocytosis assays using the protocol of Example 5.

EXAMPLE 10

Generation of apoptotic particles starting from PC12.

The PC-12 cell-line(ATCC number: CRL-1721) (Mesner P.W., Winters T.R., Green S.H.,(1992) J. Cell Biol.119:1669-1680, tends to grow in small clusters.

By addition of nerve growth factor-beta (50ng/ml final conc., Sigma), PC-12 cells differentiate into neuronal cells. By withdrawal of nerve growth factor after 5 days of treatment, programmed cell death in neuronal rat PC12 cells is induced.
The cells are cultured in RPMI 1640 (Life Technologies) with 2mM L-glutamine adjusted to contain 1.5g/L sodium bicarbonate, 4.5g/L glucose, 10mM HEPES and 1mM sodium pyruvate, 10% horse serum, 5% fetal bovine serum.

These cells can be tested for apoptotic character using the annexin/PI kit described above.

SEQUENCE LISTING

The nucleotide and amino acid sequences shown in the Figures herein are designated the following SEQ ID Nos.

SEQ ID NO: 1	Figure 1
SEQ ID NO: 2	Figure 2
SEQ ID NO: 3	Figure 3
SEQ ID NO: 4	Figure 4
SEQ ID NO: 5	Figure 5
SEQ ID NO: 6	Figure 7
SEQ ID NO: 7	Figure 9
SEQ ID NO: 8	Figure 11
SEQ ID NO: 9	Figure 17
SEQ ID NO: 10	Figure 19
SEQ ID NO: 11	Figure 26
SEQ ID NO: 12	Figure 28
SEQ ID NO: 13	Figure 31

CLAIMS:

1. An expression vector comprising a sequence of deoxynucleotides encoding a human CED-6 protein comprising the amino acid sequence of Figure 4 or Figure 5 or an amino acid sequence which differs from the amino acid sequence of Figure 4 or Figure 5 only in amino acid changes which are conservative of function.

2. An expression vector as claimed in claim 1 comprising the sequence of deoxynucleotides shown from the transcription start codon to the transcription stop codon in Figure 2 or Figure 3.

3. An expression vector as claimed in claim 1 or claim 2 which comprises a sequence of deoxynucleotides encoding a reporter gene positioned in said vector such that expression of said human CED-6 protein or functionally conserved variant thereof results in expression of a reporter protein from said reporter gene.

4. An expression vector as claimed in claim 3 wherein said reporter gene is positioned 5′ to the sequence of deoxynucleotides encoding said human CED-6 protein or functionally conserved variant thereof.

5. An expression vector as claimed in claim 3 wherein said reporter gene is positioned 3′ to the sequence of deoxynucleotides encoding said human CED-6 protein or a functionally conserved variant thereof.

6. An expression vector as claimed in any of claims 3 to 5 wherein said reporter gene encodes green flourescent protein (GFP).

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7. An expression vector as claimed in claim 4 which is pEGFP-C2 with a sequence of deoxynucleotides encoding a human CED-6 protein which comprises the sequence of amino acids as shown in Figure 4 or Figure 5 or a functionally conserved variant thereof inserted in the multicloning site.

8. An expression vector as claimed in claim 5 which is pEGFP-N3 with a sequence of deoxynucleotides encoding a human CED-6 homologue which comprises the sequence of amino acids as shown in Figure 4 or Figure 5 or a functionally conserved variant thereof inserted in the multicloning site.

9. An expression vector as claimed in claim 4 or 7 wherein said vector comprises the nucleotide sequence of Figure 28 (pGA3103).

10. An expression vector as claimed in claim 5 or 8 wherein said vector comprises the nucleotide sequence of Figure 9(pGA3104).

11. An expression vector as claimed in claim 1 or claim 2 wherein the human CED-6 protein or functionally conserved variant thereof expressed from said vector includes an epitope tag at the amino and/or the carboxy terminus thereof.

12. An expression vector as claimed in claim 11 wherein said epitope tag is His A.

13. An expression vector as claimed in claim 11 which is pBAD/HisA with a sequence of deoxynucleotides encoding a human CED-6 protein comprising the sequence of amino acids as shown in Figure 4 or Figure 5, or a functionally conserved variant thereof, inserted therein.



14. An expression vector as claimed in claim 12 which has the sequence of deoxynucleotides shown in Figure 17 (pGA 1028).

15. A mammalian cell-line transfected with an expression vector as claimed in any one of claims 1 to 13.

16. A mammalian cell-line as claimed in claim 15 wherein said cell is selected from a fibroblast cell-line or an epithelial cell line.

17. A mammalian cell-line as claimed in claim 16 wherein said cell-line is selected from COS1, BHK21, L929, CV1, SWISS 3T3, HT144, IMR32, HEPG2, MDCK, MCF7, 293, Hela, A549, SW48 or G361.

18. A mammalian cell-line as claimed in claim 15 wherein said cell-line is a primary cell-line.

19. A mammalian cell-line as claimed in claim 18 wherein said cell-line is selected from human dermal FIBs, dermal keratinocytes, leucocytes, monocytes, lymphocytes, dendritic cells or macrophages.

20. A mammalian cell-line as claimed in claim 19 which is mouse macrophage cell-line J774 or human monocyte cell-line THP-1.

21. A method for determining whether a compound is an inhibitor or an enhancer of a signal transduction pathway which promotes phagocytosis of apoptotic cells which method comprises exposing transfected mammalian cells as claimed in any one of claims 15 to 20 to apoptotic particles and measuring the rate of phagocytic uptake of said particles by



said transfected cells in the presence and absence of said compound.

22. A method as claimed in claim 21 wherein said transfected cells are exposed to said compound prior to addition of said apoptotic particles.

23. A method as claimed in claim 21 or 22 wherein said apoptotic particles are selected from the group consisting of apoptotic neutrophils, apoptotic lymphocytes and apoptotic erythrocytes which optionally have been opsonised.

24. A method as claimed in any of claims 21 to 23 wherein said apoptotic particles comprise adherent cell-line PC12.

25. A method as claimed in any of claims 21 to 23 wherein said apoptotic particles comprise the growth factor dependent mouse cell-line Ba/F3.

26. A method as claimed in claim 25 wherein said Ba/F3 cells are rendered apoptotic by culturing in the absence of growth factor IL-3.

27. A method as claimed in any one of claims 23 to 26 wherein the cells comprising said apoptotic particles are stably transfected with a reporter gene.

28. A method as claimed in claim 27 wherein said reporter gene is selected from a gene encoding β-galactosidase, a gene encoding a fluorescent protein or a gene encoding a protein capable of generating luminesence.

29. A method as claimed in claim 28 wherein

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said protein capable of generating luminescence is luciferase.

30. A method as claimed in claim 28 wherein said fluorescent protein is green fluorescent protein.

31. A method as claimed in claim 26 wherein said apoptotic particles comprises Ba/F3 cells stably transfected with β-galactosidase or luciferase.

32. A method as claimed in claim 31 wherein the level of phagocytosis is detected by adding a substrate which is converted by said β-galactosidase to a fluorescent compound.

33. A method as claimed in any one of claims 21 to 32 wherein if no phagocytosis or a reduced amount of phagocytosis is observed on exposure to the test compound, said mammalian transfected cells are examined for viability.

34. A method as claimed in claim 33 wherein if viable the phenotype of said mammalian transfected cells is compared with the phenotype of untransfected mammalian cells of the same cell-line.

35. A method as claimed in any of claims 21 to 32 wherein if an increased amount of phagocytosis is observed in the presence of the test compound, the method comprises the further steps of exposing said compound to an untransfected mammalian cell of the same cell-line and observing whether the compound induces a phenotype which is substantially the same as the phenotype exhibited by said transfected mammalian cell.

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36. A compound identified by the method of any of claims 21 to 35 as an inhibitor or an enhancer of a signal transduction pathway which promotes phagocytosis of apoptotic cells.

37. A method for determining whether a compound is an inhibitor or an enhancer of a signal transduction pathway which promotes phagocytosis of apoptotic cell which method comprises the steps of:

(1) micro-injecting into a mammalian cell a human CED-6 protein comprising the sequence of amino acids shown in Figure 4 or Figure 5 or a sequence of amino acids differing from that shown in Figure 4 or Figure 5 only in amino acid changes conservative of function.

(2) exposing the mammalian cell produced in step (1) to apoptotic particles and measuring the rate of phagocytic uptake of said particles by said cell in the presence and absence of said compound.

38. A method as claimed in claim 37 wherein said micro-injected mammalian cells are exposed to said compound prior to addition of said apoptotic particles.

39. A method as claimed in claim 38 wherein said apoptotic particles are selected from apoptotic neutrophils, apoptotic lymphocytes and apoptotic erythrocytes which optionally have been opsonized.

40. A method as claimed in any of claims 37 or 38 wherein said apoptotic particles comprise adherent cell-line PC12.

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41. A method as claimed in any of claims 37 to 39 wherein said apoptotic particles comprise the growth factor dependent mouse cell-line Ba/F3.

42. A method as claimed in claim 41 wherein said Ba/F3 cells are rendered apoptotic by culturing in the absence of growth factor IL-3.

43. A method as claimed in any one of claims 39 to 42 wherein the cells comprising said apoptotic particles are stably transfected with a reporter gene.

44. A method as claimed in claim 27 wherein said reporter gene is selected from a gene encoding β-galactosidase, a gene encoding a fluorescent protein and a gene encoding a protein capable of generating luminescence.

45. A method as claimed in claim 44 wherein said protein capable of generating luminescence is luciferase.

46. A method as claimed in claim 44 wherein said fluorescent protein is green fluorescent protein.

47. A method as claimed in claim 42 wherein said apoptotic particles comprise Ba/F3 cells stably transfected with β-galactosidase.

48. A method as claimed in claim 47 wherein the level of phagocytosis is detected by adding a substrate which is converted by said β-galactosidase to a fluorescent compound.

49. A method as claimed in any of claims 37 to



48 wherein the mammalian cell is a fibroblast cell or an epithelial cell.

50. A method as claimed in claim 49 wherein the mammalian cell is selected from COS1, BHK21, L929, CV1, SWISS 3T3, HT144, IMR32, HEPG2, MDCK, MCF7, 293, Hela, A549, SW48 or G361.

51. A method as claimed in any of claims 37 to 48 wherein said mammalian cell is a primary cell.

52. A method as claimed in claim 51 wherein said mammalian cell is selected from human dermal FIBs, dermal keratinocytes, leucocytes, monocytes or macrophages.

53. A method as claimed in claim 52 wherein said mammalian cell is a mouse macrophage cell J774 or a human monocyte cell THP-1.

54. A method as claimed in any one of claims 37 to 53 wherein if no phagocytosis or a reduced amount of phagocytosis is observed on exposure to the test compound, said mammalian transfected cells are examined for viability.

55. A method as claimed in claim 54 wherein, if viable, the phenotype of said mammalian transfected cells is compared with the phenotype of untransfected mammalian cells of the same cell-line.

56. A method as claimed in any of claims 21 to 32 wherein if an increased amount of phagocytosis is observed in he presence of the test compound, the method comprises the further steps of exposing said compound to an untransfected mammalian cell of the same cell-line and observing whether the compound

induces a phenotype which is substantially the same as the phenotype exhibited by said transfected mammalian cell.

57. A compound identified by the method of any of claims 37 to 56 as an inhibitor or an enhancer of a signal transduction pathway which promotes phagocytosis of apoptotic cells.

58. A method for determining whether a compound is an inhibitor or an enhancer of a signal transduction pathway which promotes phagocytosis of apoptotic cells which method comprises the steps of:

(1) micro-injecting or transfecting into a mammalian cell a vector expressing RNA antisense to all or a portion of the sequence of nucleotides shown in Figure 2 or Figure 3;

(2) exposing the mammalian cell produced in step (1) to apoptotic particles and measuring the rate of phagocytic uptake of said particles by said cell in the presence or absence of said compound.

59. A method as claimed in claim 58 wherein said antisense RNA comprises a sequence of nucleotides which is capable of hybridizing to a sequence of nucleotides as shown in Figure 2 or Figure 3 under conditions of stringency which are higher than 2xSSC; 0.1% SDS; 25°C to 50°C.

60. A method as claimed in claim 58 or claim 59 comprising the features of any one of claims 38 to 56.



61. A compound identified by the method claims 58 to 60 as an inhibitor or an enhancer of a signal transduction pathway which promotes phagocytosis of apoptotic cells.

62. A peptide which comprises a fragment of a human CED-6 homologue having an amino sequence as shown in Figure 4 wherein said fragment includes the sequence of amino acids NRAFSRKKDKTC, FLGSTEVEQPKGTE or TRNGTQPPPVPSRST.

63. A peptide as claimed in claim 62 consisting of the sequence of amino acids NRAFSRKKDKTC, FLGSTEVEQPKGTE or TRNGTQPPPVPSRST.

64. An antibody preparation comprising antibodies directed to one or more of the following epitopes of human CED-6 homologue as shown in Figure 4: NRAFSRKKDKTC, FLGSTEVEQPKGTE or TRNGTQPPPVPSRST

65. An antibody preparation comprising antibodies directed to the human CED-6 homologue epitope NRAFSRKKDKTC.

66. An antibody preparation comprising antibodies directed to the human CED-6 homologue epitope FLGSTEVEQPKGTE.

67. An antibody preparation comprising antibodies directed to the human CED-6 homologue epitope TRNGTQPPPVPSRST.

68. An antibody preparation as claimed in any one of claims 63 to 66 wherein said antibodies are polyclonal antibodies.

69. A method for diagnosing a disease

associated with the over or under expression of human CED-6 protein in phagocytic cells in an individual which comprises:

(a) obtaining a sample of phagocytes from said individual;

(b) exposing said phagocytes to an antibody preparation as claimed in any of claims 64 to 68;

(c) quantitatively measuring the presence of any immune complexes formed between said antibodies and said CED-6 protein; and

(d) comparing the amount of immune complex formed with that formed using phagocytes from a control individual.

70. A method for determining whether a compound is an inhibitor or an enhancer of a signal transduction pathway which promotes phagocytosis of apoptotic cells which method comprises:

(a) exposing a mammalian cell transfected with an expression vector as claimed in any one of claims 1 to 14 to the compound to be tested;

(b) exposing said mammalian cell to an antibody preparation as claimed in any of claims 64 to 68;

(c) quantitatively measuring the presence of any immune complex formed between said antibodies and protein expressed by said cells; and



(d) comparing the level of immune complex detected with the amount of immune complex detected in a mammalian cell transfected as described in step (a) which has not been exposed to said compound.

71. A method as claimed in claim 70 wherein said mammalian cell is selected from COS1, BHK21, L929, CU1 SWISS 3T3, HT144, IMR32, HEPG2, MDCK, MCF7, 293, Hela, A549, SW48 or G361.

72. A method as claimed in claim 71 wherein said mammalian cell is a COS1 cell.

73. A method as claimed in claim 70 wherein the mammalian cell is a human dermal FIB, dermal keratinocyte, leucocyte, monocyte or macrophage.

74. A method as claimed in claim 73 wherein said cell is mouse monocyte cell J774 or human monocyte cell THP-1.

75. A fusion protein which comprises:

(1) a sequence of amino acids as shown in Figure 4 or Figure 5 or a sequence of amino acids which differs from the sequence shown in Figure 4 or Figure 5 only in amino acid changes conservative of function; and

(2) a protein which is the expression product of a reporter gene.

76. A fusion protein as claimed in claim 75 which is obtained by expression of the GFP and h1ced-6 encoding sequences shown in Figures 9 or 28.

77. A fusion protein which comprises:

(1) a sequence of amino acids as shown in Figure 4 or Figure 5 or a sequence of amino acids which differs from the sequence shown in Figure 4 or Figure 5 only in amino acid changes conservative of function, and

(2) an epitope tag.

78. A fusion protein as claimed in claim 77 which is obtainable by expression of the HisA and h1ced-6 encoding sequences shown in Figure 17.

79. A method of identifying a compound which is an enhancer or an inhibitor of phagocytosis of apoptotic cells which comprises:

a) exposing a mammalian professional or semi-professional phagocyte to an apoptotic mammalian cell which has been stably transfected with a reporter gene capable of generating a signal detectable without microscopy, in the presence and absence of the compound to be tested,

b) removing any apoptotic cells which are not engulfed by said phagocytes and

c) detecting any signal of the reporter gene from said phagocytes;

wherein any difference in signal in the presence of said compound compared to the signal in the absence of said compound is an indication that said compound is an inhibitor or an enhancer of phagocytosis of apoptotic cells.

80. A method as claimed in claim 79 wherein



said phagocyte is mouse macrophage cell-line J774 or human monocyte cell-line THP-1.

81. A method as claimed in claim 80 wherein the monocyte cell-line is cultured under conditions to differentiate it into macrophages prior to exposure to said apoptotic particles.

82. A method as claimed in any of claims 79 to 81 wherein said phagocyte is a transgenic cell.

83. A method as claimed in claim 82 wherein said phagocyte has been transfected with an expression vector as claimed in any of claims 1 to 14.

84. A method as claimed in claim 82 wherein said phagocyte has been transfected with an expression vector encoding the cell surface receptor CD36.

85. A method as claimed in claim 84 wherein said phagocyte has been transfected with a vector as shown in Figure 31.

86. A method as claimed in any of claims 79 to 85 wherein said apoptotic cells comprise the adherent cell-line PC12.

87. A method as claimed in any one of claims 79 to 85 wherein said apoptotic cells comprise the growth factor dependent mouse cell-line Ba/F3.

88. A method as claimed in claim 26 wherein said Ba/F3 cells are rendered apoptotic by culturing in the absence of the growth factor IL-3.

89. A method as claimed in claim 88 wherein said cells are considered apoptotic if about 20% annexin positive and less than about 5% propidium iodide negative.

90. A method as claimed in any of claims 79 to 89 wherein said reporter gene is selected from a gene encoding β-galactosidase, a gene encoding a fluorescent protein or a gene encoding a protein capable of generating luminescence.

91. A method as claimed in claim 90 wherein said protein capable of generating luminescence is luciferase.

92. A method as claimed in claim 91 wherein said apoptotic cell has been stably transfected with a plasmid exhibiting the expression characteristics of PGL2control shown on Figure 19.

93. A method as claimed in claim 92 wherein said apoptotic cell has been stably transfected with a plasmid comprising the sequence of deoxynucleotides shown in Figure 19.

94. A method as claimed in any one of claims 79 to 89 wherein fluorescent protein is green fluorescent protein (GFP).

95. A method as claimed in claim 94 wherein said apoptotic cell has been stably transfected with a plasmid exhibiting the expression characteristics or a plasmid as shown in Figure 10 or Figure 29.

96. A method as claimed in claim 95 wherein said apoptotic cell has been transfected with a plasmid comprising the sequence of nucleotides shown

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in Figure 9 of Figure 28.

97. A method as claimed in any of claims 79 to 89 wherein said apoptotic cells have been stably transfected with a plasmid expressing b-galactosidase.

98. A method as claimed in claim 97 wherein said plasmid has the expression characteristics of the plasmid shown in Figure 11.

99. A method as claimed in claim 98 wherein said plasmid comprises the sequence of deoxynucleotides shown in Figure 11.

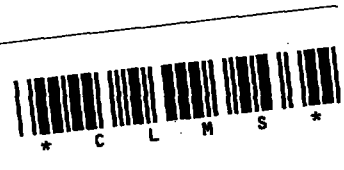
100. A method as claimed in any of claims 79 to 89 wherein said apoptotic particles comprise cell-line Ba/F3 stably transfected with β-galactosidase.

101. A method as claimed in claim 100 wherein the level of phagocytosis is detected by adding a substrate which is converted by said b-galactosidase to a fluorescent compound.

102. A method as claimed in any one of claims 79 to 101 wherein if no phagocytosis or a reduced amount of phagocytosis is observed on exposure to the test compound, said phagocytes are tested for viability.

103. A method as claimed in any of claims 79 to 102 wherein said phagocytes are cultured in multiwell plates the apoptotic cells and the test compounds being added to the individual wells thereof.

104. A method as claimed in any preceding claim



wherein the signal from said reporter gene is detected by an automatic plate reader.

105. A method as claimed in claim 101 wherein the signal from the reporter gene is detected by an automatic plate reader capable of detecting a fluorescent signal.

106. A compound identified as an inhibitor or enhancer of phagocytosis of apoptotic cells by the method of any claims 79 to 105.

107. A method as claimed in any of claims 22 to 35, 39 to 56, and 58 to 60 wherein phagocytic uptake is measured by non-microscopic means.

108. A method as claimed in claim 107 wherein said non-microscopic means is a multi-well plate reader.

109. A method as claimed in claim 108 wherein said multi-well plate reader measures luminescence. fluorescence or performs spectrophotometric detection.

110. A method as claimed in any of claims 79 to 105 having the features of claims 108 and 109.

111. A method for diagnosing a disease associated with the over- or under-expression of human CED-6 in phagocytic cells in an individual, which method comprises:

(a) obtaining a sample of phagocytes from said individual,

(b) isolating RNA from said phagocytes,

(c) preparing cDNA from said RNA,

(d) performing a first PCR reaction on said



cDNA,

(e) performing a second (nested) PCR on the reaction product of said first PCR reaction,

(f) quantitatively and qualitatively measuring the presence of CED-6 RNA by analysing the reaction products from the first and second PCR,

(g) comparing the amount and type of reaction products formed in the first and second PCR with that of the reaction products formed using phagocytes from control individuals.

112. A method as claimed in claim 111 wherein said PCR is performed with primers derived from the sequence of human CED-6, or derived from the vector used in the generation of cDNA.

113. A method as claimed in claim 111 or 112 wherein said first PCR is performed with primers having nucleotide sequences:

1) cgcaaggatccccatgaaccgtgcttttagcaggaag

2) gatctactaggtactggag

114. A method as claimed in any of claims 111, 112 or 113 wherein said second PCR is performed with primers having nucleotide sequences:

1) cgcaaggatccccatgaaccgtgcttttagcaggaag

2) gcggatggtaccgtcgactgctgatacttgagttattctcag

