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(54) Titre : ENTITES APOPTOTIQUES POUR LE TRAITEMENT DE TROUBLES DE DYSFONCTIONNEMENT DE L'ENDOTHELIUM

(54) Title: APOPTOTIC ENTITIES FOR USE IN TREATMENT OF ENDOTHELIUM DYSFUNCTION DISORDERS

(57) Abrégé/Abstract:
Treatment and/or prophylaxis, in mammalian patients, of medical disorders associated with a dysfunctional endothelium, is effected by administering to the patient effecive amounts of apoptotic bodies and/or apoptotic cells, preferably those derived from the patient's own white blood cells, e.g. by extracorporeal treatment of the patient's blood cells to induce apoptosis and administration of the apoptotic bodies and/or cells so formed to the patient.

ABSTRACT OF THE DISCLOSURE

Treatment and/or prophylaxis, in mammalian patients, of medical disorders associated with a dysfunctional endothelium, is effected by administering to the patient effecive amounts of apoptotic bodies and/or apoptotic cells, preferably those derived from the patient's own white blood cells, e.g. by extracorporeal treatment of the patient's blood cells to induce apoptosis and administration of the apoptotic bodies and/or cells so formed to the patient.

APOPTOTIC ENTITIES FOR USE IN TREATMENT OF ENDOTHELIUM DYSFUNCTION DISORDERS.

Field of the Invention

This invention relates to biochemical and biological compositions and to the uses thereof in the treatment and/or prophylaxis, in mammalian patients, of various medical disorders associated with endothelial dysfunction (Malfunctioning of the lining of blood vessels). More particularly, it relates to treatment and prophylaxis of medical disorders associated with endothelial dysfunction by administration of compositions containing mammalian cellular materials and fragments thereof, and to the compositions containing the mammalian cellular materials and fragments themselves, and to processes for preparing such compositions.

Background of the Invention

Two mechanisms of cell death in the body are recognized, necrosis and apoptosis. Apoptosis is the process of programmed cell death, first described by Kerr et al in 1972 [Kerr JFR, Wyllie AH, Currie AR (1992). "Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *"British Journal of Cancer* **26:** 239-257", by which steady-state levels of the various organ systems and tissues in the body are maintained as continuous cell division and differentiation takes place. Cells undergoing apoptosis often exhibit distinctive morphological changes such as pronounced decrease in cell volume, modification of the cytoskeletons resulting in pronounced membrane blebbing, a condensation of the chromatin, and degradation of the DNA into oligonucleosomal fragments. Following these morphological changes, an apoptotic cell may break up into a number of small fragments known as apoptotic bodies, consisting essentially of membrane-bound bodies containing intact organelles, chromatin etc. Apoptotic bodies are normally rapidly removed from the body by phagocytosis principally by

macrophages, before they can become lysed and release their potentially pro-inflammatory intracellular contents.

In simple outline, apoptosis is thought to proceed as follows. Three phases can be identified in the apoptotic mechanism of programmed cell death:

Induction phase

Effector phase

Degradation phase.

The induction phase is dependent on specific interactions of death-inducing signals at the cell surface membrane. One common signal is initiated by the binding of specific ligands to receptors of the TNF receptor family present on the cell membrane. One important such receptor is Fas (APO-1, CD95), which interacts with Fas-ligand to initiate apoptosis.

The effector phase, activated by the binding of receptors and ligands of the induction phase, leads to the activation of caspases, cystinyl-aspartate-requiring proteinases (proteolytic enzymes), including caspases 1 and 8. This activation is associated with a change in the permeability of mitochondria, allowing the release of cytochrome-c which is involved in caspase activation. Activated caspases initiate a chain of lethal proteolytic events culminating in the changes in chromatin and cytoskeletal components seen in apoptosis.

Many cells undergoing apoptosis can be identified by a characteristic 'laddering' of DNA seen on agarose gel electrophoresis, resulting from cleavage of DNA into a series of fragments. These changes occur a few hours before death of the cell as defined by the ability of a cell to exclude vital dyes. The appearance of DNA laddering on agarose gel electrophoresis following extraction of DNA from cells is one recognized method of identification of apoptosis in cells [Loo, D.T. and Rillema, J.R. (1998) "Measurement of Cell

Death," *Methods in Cell Biology* **57**: 251-264], although it is not always sensitive enough to detect apoptosis. *In situ* labelling of nuclear DNA fragmentation for example using commercially available terminal dUTP nick end labelling (TUNEL) assays, are an alternative and more reproducible measure for the determination of fragmented DNA in apoptotic cells and cells undergoing apoptosis [Gavrieli Y, Sherman Y, Ben-Sasson SA (1992)", Identification of programmed cell death in situ via specific labelling of nuclear DNA fragmentation". *Journal of Cell Biology* **119:** 493-501].

During apoptosis, phosphatidylserine becomes exposed externally on the cell membrane [Fadok VA, Voelker DR, Campbell PA, Cohen JJ, Bratton DL, Henson PM (1992), "Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages". *Journal of Immunology* **148:** 2207-2216] and this exposed phosphatidylserine binds to specific receptors to mediate the uptake and clearance of apoptotic cells in mammals [Fadok VA, Bratton DL, Rose DM, Pearson A, Ezekewitz RAB, Henson PM (2000), "A receptor for phosphatidylserine-specific clearance of apoptotic cells", *Nature* **405:** 85-90]. The surface expression of phosphatidylserine on cells is another recognized method of identification of apoptotic cells.

Changes in mitochondrial integrity are intimately associated with apoptosis, resulting in alterations in mitochondrial membrane permeability and the release of cytochrome-c from the mitochondria into the cell cytoplasm [Susin, S.A., Lorenzo, H.K., Zamzami, N., Marzo, I, Brenner, C., Larochette, N., Prevost, M.C., Alzari, P.M. and Kroemer, G. (1999) "Mitochondrial Release of Caspase-2 and -9 during the Apoptotic Process", *Journal of Experimental Medicine,* **189**: 381 - 394]. Measurement of changes in mitochondrial membrane potential, reflecting changes in mitochondrial membrane permeability, is another recognized method of identification of apoptotic cells.

A number of other methods of identification of cells undergoing apoptosis and of apoptotic cells, many using monoclonal antibodies against specific markers for apoptotic cells, have also been described in the scientific literature.

Necrosis, in contrast, is cell death of a pathological nature, resulting from injury, bacterial toxin effects, inflammatory mediators, etc., and involving membrane rupture and release of intracellular contents to the surrounding tissue, often with harmful inflammatory consequences.

Summary of the Invention

According to the present invention, the administration of apoptotic cells and/or apoptotic bodies previously prepared *ex vivo* are used in the prophylaxis and treatment of medical disorders in which there is dysfunction of the cells of the endothelium, the cellular lining of blood vessels.

The endothelium is a highly specialized interface between blood and underlying tissues and has a number of functions including:

control of haemostasis by inhibiting platelet aggregation (anti-thrombotic and regulating the coagulation and fibrinolytic systems;

control of vascular tone;

control of blood vessel smooth muscle growth;

selective permeability to cells and proteins.

Normally, the endothelium maintains vascular homeostasis by responding to physiological stimuli, for example changes in blood flow, oxygen tension etc., by adaptive alteration of function. Dysfunctional endothelium has an impaired response to such physiological stimuli, and can ultimately lead to medical disorders. A number of subsets of endothelial dysfunction have been recognized, including Endothelial Activation, and Endothelial-mediated Vasodilatory Dysfunction (see De Caterina (2000). "Endothelial dysfunctions:

common denominators in vascular disease". Current Opinions in Lipidology 11:9-23).

Endothelial Activation may lead to the initiation of atherosclerosis and is a process whereby there is an inappropriate up-regulation and expression of cell attraction and cell adhesion molecules on endothelial cells. This particularly involves the Macrophage Chemoattractant Protein-1 (MCP-1), chemoattractants for lymphocytes (IP-10, MIG, I-TAG), and the Bascular Cell Adhesion Molecule-1 (VCAM-1), to which the monocytes and lymphocytes adhere. Once adherent, the leucocytes enter the artery wall. The monocytes and lymphocytes are recruited to the intima (sub-endothelial layers) of the blood vessels by these cell attraction and cell adhesion molecules of the activated endothelium during the early stages of atherosclerosis (see Libby P (2000). "Changing concepts of atherogenesis". *Journal of Internal Medicine 247:349-358.*

Endothlial-mediated Vasodilatory Dysfunction, is characterized by a reduction or loss of endothelium-dependent vasodilation and involves "decreased nitric oxide bioavailability" (decreased production, increased destruction and/or decreased sensitivity to nitric oxide). [De Caterina (2000), cited above]. Nitric oxide induces vasodilation by relaxing the smooth muscle cells of the blood vessel wall. Endothelial-mediated Vasodilatory Dysfunction can be measured as a reduction in vasodilation in response in response to acetylcholine, or as a reduced in vasodilatory response following occlusion of arterial blood flow for example using a sphygmomanometer cuff. As well as leading to a reduction in vasodilation, decreased endothelial nitric oxide bioavailability can also result in an increase in the production of vaso-constriction and hypertension. Platelet aggregation is potently inhibited by nitric oxide, hence a decrease in nitric oxide bioavailability can lead to an increase in platelet aggregation and consequent thrombosis. These are just a few examples of how decreased nitric oxide bioavailability resulting from

Endothelial-mediated Vasodilatory Dysfunction can have pathological consequences.

The medical disorder resulting from endothelial dysfunction can be a cardiovascular disorder such as atherosclerosis, peripheral vascular disease, congestive heart failure, stroke, myocardial infarction, angina, hypertension etc. It can be a vasospastic disorder such as Raynaud's disease, cardiac syndrome X, migraine etc. It can be the damage resulting from ischemia (ischemic injury or ischemia-reperfusion injury). In summary, it can be substantially any disorder that results from an inappropriately functioning endothelium.

"Apoptotic cells and apoptotic bodies", as the term is used herein, means cells and cell bodies which exhibit one or more of the following apoptosis-characterizing features:

surface exposure of phosphatidylserine, as detected by standard, accepted methods of detection such as Annex in V staining, methods for which are commercially available (for example, Annex in V-FTIC kit, Stress-Gen Biotechnologies Corp, Vancouver, Canada);

alterations in mitochondrial membrane permeability measured by standard, accepted methods (e.g. Salvioli, S., Ardizzoni, A., Franceschi, C. Cossarizza, A. (1997) "JC-1, but not DiOC6(3) or Rhodamine 123, is a Reliable Fluorescent Probe to assess Delta Psi Changes in Intact Cells: Implications for Studies on Mitochondrial Functionality during Apoptosis," *FEBS Letters* **411:** 77-82];

evidence of DNA fragmentation such as the appearance of DNA laddering on agarose gel electrophoresis following extraction of DNA from the cells [Teiger, E., Dam, T.V., Richard, L., Wisnewsky, C., Tea, B.S., Gaboury, L., Tremblay, J., Schwartz, K. and Hamet, P. (1996) "Apoptosis in Pressure Overload-induced Heart Hypertrophy in the Rat," *Journal of Clinical*

Investigation **97**; 2891-2897], or by *in situ* labeling (see Gavrieli et al., 1992, referenced above).

Description of the Preferred Embodiments

The apoptotic cells and/or apoptotic bodies for use in the present invention are previously prepared *ex vivo* from mammalian cells that are compatible with those of the mammalian patient. They can be prepared from substantially any type of mammalian cell including cultured cell lines. Preferably they are prepared from a cell type derived from the mammalian patient's own body or from an established cell line. More preferably they are prepared from white blood cells of blood compatible with that of the mammalian patient, even more preferably from the patient's own white blood cells and most preferably from the patient's own T lymphocytes. The apoptotic cells and/or apoptotic bodies are prepared extracorporeally prior to administration to the patient. Thus, an aliquot of the patient's blood may be withdrawn, e.g. by venipuncture, and at least a portion of the white cells thereof subjected extracorporeally to apoptosis inducing conditions.

A variety of methods of inducing apoptosis in mammalian cells, so as to create apoptotic cells and apoptotic bodies, are known in the art and essentially any of these can be adopted in preparing apoptotic bodies for use in the present invention. One such method is the application of oxidative stress to cells extracorporeally (see for example Buttke and Sandstrom (1994) "Oxidative Stress as a Mediator of Apoptosis", Immunology Today, Vol. 15:7-10). This can be achieved by treating the cells, in suspension, with chemical oxidizing agents such as hydrogen peroxide, other peroxides and hydroperoxides, ozone, permanganates, periodates, and the like. Biologically acceptable such oxidizing agents are preferably used, so as to reduce potential problems associated with residues associated with and contaminating the apoptotic cells and apoptotic bodies so formed. Another method is the subjection of the cells to ionizing radiation (γ-rays, x-rays, etc.) and/or non ionizing electromagnetic radiation

including ultraviolet light. Apoptosis can be induced by subjecting cells to ultrasound. Yet another method is the treatment of the cells with drugs such as non-specific protein kinase inhibitors as exemplified by staurosporine (see Bombeli, Karsan, Tait and Hirlan, (1997) "Apoptotic Vascular Endothelial Cells Become Procoagulant", Blood, Vol. 89:2429-2442). Also, certain chemotherapeutic agents used for the treatment of malignant tumours induce apoptosis, for example adriamycin, as can statin drugs (3-hydroxy-3methylglutaryl coenzyme A reductase inhibitors) [Guijarro C, Blanco-Colio LM, Ortego M, Alonso C, Ortiz A, Plaza JJ, Diaz C, Hernandez G, Edigo J (1998), "3-hydroxy-3methylglutaryl coenzyme A reductase and isoprenylation inhibitors induce apoptosis of vascular smooth muscle in culture". "*Circulation Research* **83:** 490-500] and colcicine [Suzuki Y (1998)", "Cell death, phagocytosis and neurogenesis in mouse olfactory epithelium and vomeronasal organ after colcicine treatment". *Annals of the New York Academy of Sciences* **855:** 252-254]. The use of ligands for death receptors on cells, such as Fas-ligand, will be apparent for inducing apoptosis from the discussion of apoptosis above. The present invention is not restricted to any particular method of producing apoptotic cells and apoptotic bodies, for use in the present invention, and any suitable, known process can be used.

Methods for the detection and quantitation of apoptosis can be used to determine the presence and level of apoptosis in the preparation to be administered to the patient in the present invention. At least one of the methods from those described in the Introduction above should be used to confirm the level of apoptosis achieved prior to administration.

In preparing the apoptotic bodies, care should be taken not to apply excessive levels of oxidative stress, radiation, drug treatment, etc., since otherwise there is a significant risk of causing necrosis of at least some of the cells under treatment. Necrosis causes cell membrane rupture and the release of cellular contents often with biologically harmful results, particularly inflammatory events, so that the presence of necrotic cells and their

components along with the apoptotic bodies is best avoided. -The process of apoptosis should be conducted under conditions which cause apoptosis, or other inactivation or down-regulation, of the potentially phagocytosing cells present in the cellular composition under treatment, such as macrophages, since otherwise the apoptotic bodies produced are liable to be phagocytosed before administration to the patient, and thereby rendering the preparation less effective. Appropriate levels of treatment of the cells to create apoptotic bodies for use in the present invention depend to some extent on the nature of the chosen cells and cellular composition, and the type of treatment chosen to induce apoptosis. Such appropriate levels are readily determinable by those skilled in the art, having regard to the available scientific literature on the subject including the above-reference articles.

One preferred process according to the present invention involves the culture of cells from the patient, or a compatible mammalian cell line. The cultured cells may then be treated to induce apoptosis and create apoptotic cells and/or apoptotic bodies therein. The cells, suspended in the patient's plasma or another suitable suspension medium, such as saline or a balanced mammalian cell culture medium, can then be administered as indicated below. The numbers of apoptotic cells can be determined by published methods available in the scientific literature on the subject including the above-reference articles. The numbers of such apoptotic cells and/or apoptotic bodies required for administration to the patient to obtain the required clinical benefit will vary depending on the source of cells, the patients condition etc. and may require some experimentation but are readily determinable by those skilled in the art.

A more preferred process according to the present invention accordingly involves extraction of an aliquot of blood from the patient to be treated, and treatment of the white cells thereof under apoptosis-causing conditions, so as to create a cellular composition in which significant numbers of the white cells therein have been apoptosed so as to create therein substantial numbers of apoptotic bodies. Then the treated composition is re-

administered to the patient. The aliquot treated to cause apoptosis may be whole blood, but if preferably a separated white cell fraction thereof, separated from the blood by known means, and suspended in plasma or another suitable suspension medium, such as saline or a balanced mammalian cell culture medium. More preferably, T lymphocytes, isolated from the blood by known means, and suspended as above, may be used as a source of apoptotic cells and apoptotic bodies.

The volume of the aliquot of blood withdrawn from the patient for treatment to create apoptotic cells and/or apoptotic bodies therein is suitable up to about 400 ml, preferably from about 0.1 to about 100 ml, and most preferably from about 5 to about 15 ml. Accordingly, the preferred amounts of apoptotic cells and/or apoptotic bodies for administration are those corresponding to the numbers derivable from the white blood cells, or isolated T lymphocytes, contained in such quantities of whole blood, following subjection to apoptosis-inducing conditions.

The suspension of apoptotic cells and/or apoptotic bodies, is prepared in a biologically acceptable liquid suspending medium, such as the patient's serum or plasma, saline or balanced mammalian cell culture medium. The addition of other factors, such as cytokines, hormones, products of stressed cells or other appropriate biologically active material, including cells, may enhance the benefit of the administered apoptotic cells and/or apoptotic bodies. The aliquot can be re-introduced into the patient's body by any suitable method, most preferably intramuscular injection but also including subcutaneous injection, mini-grafting, intra peritoneal injection, intra-arterial injection, intravenous injection and oral administration. The apoptotic entities can be delivered to the specific body organ and/or site by using any appropriate delivery system including liposomes, microspheres, etc.

For most effective treatment and prophylaxis of mammalian disorders involving endothelial dysfunction, the patient may be given a course

of treatments with apoptotic cells and/or apoptotic bodies according to the invention. Each course of treatment may involve administration to the patient of from 1 to 6 aliquots of suspended apoptotic cells and/or apoptotic bodies, as described above. No more than one such aliquot should be administered per day, and the maximum rest period between any two consecutive administrations should be not greater than about 21 days. Booster treatments as described below may advantageously be used. To maintain the desired effects, the patient may undergo booster treatments, with a further course of administration of aliquots of suspended apoptotic cells and/or apoptotic bodies as described above, at intervals of three to four months.

As noted, the present invention is applicable to the treatment and prophylaxis of a wide variety of mammalian disorders that involve endothelial dysfunction. These include, but are not limited to, cardiovascular diseases, such as atherosclerosis, peripheral vascular disease, congestive heart failure, stroke, myocardial infarction, angina, hypertension, etc. vasospastic disorders such as Raynaud's disease, cardiac syndrome X, migraine etc.; and the damage resulting from ischemia (ischemic injury or ischemia-reperfusion injury). In summary, it can be substantially any disorder that results from an inappropriately functioning endothelium.

WHAT IS CLAIMED IS:

1. The use of apoptotic bodies and/or apoptotic cells in treatment and/or prophylaxis in mammalian patients of medical disorders resulting from or involving endothelial dysfunction.

2. The use of apoptotic bodies and/or apoptotic cells in the preparation of a medicament for the treatment and/or prophylaxis of medical disorders resulting from or involving endothelial dysfunction, in mammalian patients.

3. Method for the treatment of or prophylaxis against medical disorders associated with endothelial dysfunction, in a mammalian patient, which comprises administering to the patient an effective amount of apoptotic bodies and/or apoptotic cells.

4. Uses and methods according to any preceding claim wherein the apoptotic bodies and/or cells derive from extracorporeal treatment of blood cells compatible with those of the mammalian patient.

5. Uses and methods according to claim 4 wherein the blood cells are white blood cells of blood compatible with that of the mammalian patient.

6. Uses and methods according to claim 5 wherein the blood cells are the patient's own white blood cells.

7. Uses and methods according to claim 6 wherein the blood cells are the patient's own T lymphocytes.