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(54) Title: SELECTIVE REMOVAL OF CELLS HAVING ACCUMULATED AGENTS

(57) **Abstract**: A method of treating a condition associate with accumulation of an agent in cells in a patient includes exposing the cells to ultrasound, to selectively kill or induce apoptosis in the cells. The cells include the accumulated agent.

SELECTIVE REMOVAL OF CELLS HAVING ACCUMULATED AGENTS

BACKGROUND

[01] Accumulation of agents, such as proteins, lipids, bacteria, viruses, parasites or particles, leads to, or is associated with, pathological conditions. For example, nucleolin, a protein normally expressed in the nucleus or the cytoplasm has been shown to be expressed at the cell surface in neoplastic cells and endothelial cells of angiogenic vessels *in vivo*. Mi Y, *et al.* Apoptosis in leukemia cells is accompanied by alterations in the levels and localization of nucleolin. *J Biol Chem* 278:8572-9 (2003); Sven C, *et al.* Nucleolin Expressed at the Cell Surface is a Marker of Endothelial Cells in Angiogenic Blood Vessels, *Journal of Cell Biology*, Vol. 164, No. 4, 871-878 (2003). Another example is P-glycoprotein (P-gp), a plasma membrane protein, which is over expressed in tumor cells that present a multidrug resistance (MDR) phenotype, which causes efflux of several structurally unrelated therapeutic drugs used for cancer treatment. Loo TW, *et al.* Identification of Residues in the Drug Translocation Pathway of the Human Multidrug Resistance P-glycoprotein by Arginine Mutagenesis, *Journal of Biological Chemistry,* Vol. 284, No. 36, 24074-24087 (2009). Deposition and subsequent accumulation of intracellular protein aggregates has been observed in several neurodegenerative disorders, such as α-synuclein in Parkinson's disease, β-amyloid and tau in Alzheimer's disease, and huntingtin in Huntington's diseases, and prion protein (PrP) in transmissible prion encephalopathies. Brandin P, *et al.* Prion-Like Transmission of Protein Aggregates in Neurodegenerative Diseases, *Nat Rev Mol Cell Biol.* Vol. 11, No. 4, 301-307 (2010). Poly A binding protein (PABP) accumulates in the cytoplasm of beta herpesviruses (HCMV)-infected cells. Perez C, *et al.* Translational control of cytoplasmic poly A binding protein (PABP) abundance in HCMV-infected cells, *J Virol.* Oct 27 (2010) Epub.

[02] Intracellular lipids accumulation is commonly observed in advanced atherosclerotic plaques. Monocyte infiltration in the intima layer of the vascular wall is followed by differentiation into macrophages, which in turn take up modified lipoproteins and become macrophage foam cells as a result of such intracellular

lipids accumulation. Persson J, *et al.* Interleukin-1 beta and tumour necrosis factor-alpha impede neutral lipid turnover in macrophage-derived foam cells, *BMC Immunology*, 9(7) (2008). Obesity is associate with the accumulation of lipids in fat cells.

[03] Some bacteria may accumulate inside cells, for example *Mycobacterium tubercolosis* and *Pseudomonas aeruginosa*. *M. tubercolosis* causes the formation of hard nodules or tubercles in the lungs, parasitizes macrophages by blocking the phagosome-lysosome fusion, a process called phagosome maturation arrest, and by replicating inside the phagosome. Vergne I, *et al.* Cell Biology of *Mycobacterium tubercolosis* Phagosome, *Ann Rev Cell Dev Biol.*, Vol. 20, 367-94 (2004). Similarly, *P. aeruginosa* colonizes the lungs of patients with cystic fibrosis and produces biofilms, alginates, and specific lipid A modifications, which allow the bacteria to escape immune response and cause severe chronic inflammation. Moskowitz SM, *et al.* The Role of *Pseudomonas* Lipopolysaccharide in Cystic Fibrosis Airway Infection, *Subcell Biochem.*, Vol. 53, 241-53 (2010). Production of biofilms by *Haemophilus influenzae*, *Streptococcus pneumoniae*, and other bacteria, has been linked to chronic otitis media in pediatric patients. Hall-Stoodley L, *et al.* Direct Detection of Bacterial Biofilms on the Middle-Ear Mucosa of Children With Chronic Otitis Media, *JAMA*, Vol. 256, No. 2, 202-11 (2006).

[04] Some protozoan parasites present intracellular accumulation, for example *Plasmodium*, *Leishmania*, *Trypanosoma* and *Toxoplasma*. *Plasmodium*, the agent causing malaria, replicates and accumulates inside erythrocytes, provoking cell rupture and dissemination of the agent, while the main sites of sequestration of the infected erythrocytes containing the trophozoites, schizonts and gametocytes of the parasite have been shown to be the lung, spleen, and adipose tissue, but also the brain, skin, bone marrow, and skeletal and cardiac muscle. Franke-Fayard B, *et al.* Sequestration and Tissue Accumulation of Human Malaria Parasites: Can We Learn Anything from Rodent Models of Malaria?, *PLoS Pathogens*, Vol. 6, No. 9, e1001032 (2010). Similarly, *Leishmania mexicana* and *Trypanosoma cruzi* reside and proliferate inside macrophages. Zhang S *et al.* Delineation of Diverse Macrophage

Activation Programs in Response to Intracellular Parasites and Cytokines, *PLoS Negl Trop Dis*, Vol. 4, No. 3: e648 (2010).

[05] Viruses replicate in the host cell, and the accumulation of the viral particles may result in changes to the plasma membrane. Examples include HIV, hepatitis C and rhinovirus. Ma Y, *et al.* NS3 helicase domains involved in infectious intracellular hepatitis C virus particle assembly, J Virol. 82 (15) 7624-39 (2008); Korant BD, Butterworth BE, Inhibition by zinc of rhinovirus protein cleavage: interaction of zinc with capsid polypeptides, *J Virol.* 18(1):298-306 (1976).

[06] Ultrasound is a technique that may be used to destroy or induce apoptosis of cells. U.S. Patent No. 6,821,274 (2004). The technique has been used to selectively remove or kill cells based on differences is the membrane stiffness, such as that caused by cross-linking from AGE-modification. The ultrasound is targeted to harmonic frequencies of the cross-linked cell membranes or components. International Publication No. WO2009/143411 (2009).

SUMMARY

[07] In a first aspect, the present invention is a method of treating a condition associate with accumulation of an agent in cells in a patient comprising exposing the cells to ultrasound, to selectively kill or induce apoptosis in the cells. The cells include the accumulated agent.

[08] In a second aspect, the present invention is a method of removing cells from a sample, comprising exposing the sample to ultrasound, to selectively kill or induce apoptosis in the cells. The cells comprise an accumulated agent.

DETAILED DESCRIPTION

[09] The present invention makes use of the discovery that the differential resonant frequency of a cell caused by the accumulation of at least one agent that causes, or is associated with, a pathological or undesired condition, such as proteins, lipids, bacteria, viruses, parasites or particles, may be used to distinguish

and eliminate cells in which the accumulated agent leads to a difference in the resonant frequency of the cell, by applying ultrasound treatment. The cells associated with the accumulated agent have a resonant frequency which is distinct from cells of the same type. By selecting the frequency of the ultrasound applied to the tissue to feed energy into the resonant frequency, the cells with the accumulated agent will be destroyed or induced to undergo apoptosis. In an aspect of the invention, the cells are not AGE-modified cells. In another aspect of the invention, the cells are not tumor cells. In yet another aspect of the invention, the cells are not cancerous.

[10] The ultrasound technique for removing cell-associated accumulation from a patient is selected for its ability to selectively kill or induce apoptosis in cells having accumulation of the agent associated with the pathological condition, while avoiding removal or destruction of cells that do not present the accumulation. For example, cells expressing high levels of nucleolin on the plasma membrane of the cell may be selected due changes in the stiffness and deformability of the cell. As used herein, “selectively kill or induce apoptosis” means that more of the cells which are the target of the killing or inducing apoptosis are so affected, as compared to other cell subject to the same exposure.

[11] Ultrasound devices can be used according to practices well known to those skilled in the art to destroy cells by vibrational techniques, for example U.S. Patent No. 5,601,526 (1997) and International Publication No. WO2009/143411 (2009). Ultrasound parameters, such as frequency, power and pulsation, can be screened for effectiveness in selectively destroying the targeted cells. Differential destruction or inducement of apoptosis may be by selection of the stiffer cells, or by selection of the cells by their resonant frequencies. Ultrasound as described above can be applied to a subject with monitoring to determine that inflammatory responses such as fever or swelling do not exceed limits well known to be safe. This process can be repeated at intervals to maintain a level of therapeutic benefit. Evaluation of improvement or maintenance of a desired result can be used to direct the frequency of reapplication of ultrasound according to the present invention. The application and

reapplication can be determined with the goal of gradual improvement to avoid overwhelming natural mechanisms, such as removal of cells and cellular debris by scavenging cells.

[12] A variety of techniques are available to determine whether ultrasound may be used to selectively remove or kill the cells having the accumulated agent, that leads to, or is associated with, a pathological or undesired condition. The stiffness of individual cells may be determined, by techniques such as those described in U.S. Patent No. 6,067,859 (2000). Elastic properties of tissue may be measured, by techniques such as those described in U.S. Patent No. 7,751,057 (2010). Furthermore, application of a variety of ultrasound parameters to cells or a tissue sample, followed by examination of the cells or the tissue sample for destruction or subsequent apoptosis, may also be used to determine whether ultrasound may selectively remove or kill the cells.

[13] EXAMPLES

[14] Example 1 (Prophetic) Ultrasound removal of cell-associated accumulation of nucleolin in leukemia cells

[15] Blood of a patient containing leukemia cells expressing nucleolin on the cell surface is treated with ultrasound. After first diagnosing the patient, a blood sample is taken for further analysis. Leukemia cells are isolated from the blood sample, and examined using an optical stretcher (U.S. Patent No. 6,067,859), to determine the relative stiffness of the cells and/or the resonant vibrational frequencies of the cell. This information is then used to select driving frequencies, intensity and length of time of treatment of the ultrasound, to selectively destroy or induce apoptosis, in the leukemia cells *in vivo* or *ex vivo*.

[16] Example 2 (Prophetic) Ultrasound removal of cell-associated accumulation of P-glycoprotein in colon cancer cells

[17] Colon tissue from a patient containing colon cancer cells that present a multidrug resistance (MDR) phenotype are examined and determined to be

overexpressing P-glycoprotein on the cell surface. The cells are then examined using an optical stretcher (U.S. Patent No. 6,067,859), to determine the relative stiffness of the cells and/or the resonant vibrational frequencies of the cell. This information is then used to select driving frequencies, intensity and length of time of treatment of the ultrasound. For example, an ultrasound generating probe may be included at the tip of a colonoscopy device. The ultrasound generating probe could generate pulses of ultrasound at the appropriate frequency, to selectively destroy colon cancer cells that present a multidrug resistance (MDR) phenotype, after the probe has been placed proximate to the tumor. The success of the treatment is monitored by subsequent colonoscopy.

[18] Example 3 (Prophetic) Ultrasound removal of cell-associated accumulation of *Plasmodium*.

[19] Blood of a patient, containing erythrocytes infected with *Plasmodium*, is treated with ultrasound. After first diagnosing the patient, a blood sample is taken for further analysis. Erythrocytes infected with *Plasmodium* are isolated from the blood sample, and examined using an optical stretcher (U.S. Patent No. 6,067,859), to determine the relative stiffness of the erythrocytes and/or the resonant vibrational frequencies of the erythrocytes. In addition, the *Plasmodium* parasite could also be examined using the optical stretcher, to determine ultrasound parameters capable of direct destruction of the parasite. This information is then used to select driving frequencies, intensity and length of time of treatment of the ultrasound, to selectively destroy infected erythrocytes and/or the *Plasmodium* parasites, in the patient's blood either *in vivo* or *ex vivo*.

[20] Example 4 (Prophetic) Removal of macrophages and tubercles infected with *Mycobacterium tubercolosis*

[21] A biopsy of an area in the lung of a patient containing tubercles and macrophages infected with *M. tubercolosis* is taken. The biopsy is treated with ultrasound applied at a range of frequencies and intensities, to determine conditions necessary to selectively destroy or induce apoptosis in the infected marcophages, the tubercles and/or the *M. tubercolosis* bacterial cells. The lungs of the patient are then treated with ultrasound. Time of exposure may range from three to sixty minutes daily for up to 20 days. At the end of the treatment, the patients are tested to determine the reduction in the size and/or number of tubercles present in the lungs of the patient.

[22] Example 5 (Prophetic) Removal of alginates caused by *Pseudomonas aeruginosa* infection in the lungs of patients with cystic fibrosis

[23] An alginate sample from the lungs of the patient is treated with ultrasound applied at a range of frequencies and intensities, to determine conditions necessary to selectively destroy or break down the alginate. The lungs of the patient are then treated with ultrasound. Time of exposure may range from three to sixty minutes daily for up to 20 days. At the end of the treatment, the patient is tested to determine the reduction of alginates in the lungs.

[24] REFERENCES

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[33] 9. Hall-Stoodley L, *et al.*, Direct Detection of Bacterial Biofilms on the Middle-Ear Mucosa of Children With Chronic Otitis Media, *JAMA*, Vol. 256, No. 2, 202-11 (2006).

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[35] 11. Zhang S, *et al.* Delineation of Diverse Macrophage Activation Programs in Response to Intracellular Parasites and Cytokines, *PLoS Negl Trop Dis*, Vol. 4, No. 3: e648 (2010).

[36] 12. Ma Y, *et al.* NS3 helicase domains involved in infectious intracellular hepatitis C virus particle assembly, J Virol. 82 (15) 7624-39 (2008).

[37] 13. Korant BD, Butterworth BE, Inhibition by zinc of rhinovirus protein cleavage: interaction of zinc with capsid polypeptides, *J Virol.* 18(1):298-306 (1976).

[38] 14. U.S. Patent No. 6,821,274 (2004).

[39] 15. International Publication No. WO2009/143411 (2009).

[40] 16. U.S. Patent No. 5,601,526 (1997).

[41] 17. U.S. Patent No. 6,067,859 (2000).

[42] 18. U.S. Patent No. 7,751,057 (2010).

What is claimed is:

1. A method of treating a condition associate with accumulation of an agent in cells in a patient, comprising exposing the cells comprising the accumulated agent to ultrasound, to selectively kill or induce apoptosis in the cells.

2. The method of claim 1, wherein the cells are not AGE-modified cells.

3. The method of any of the preceding claims, wherein the agent is selected from the group consisting of proteins, lipids, bacteria, viruses and parasites.

4. The method of any of the preceding claims, wherein the cells are not tumor cells.

5. The method of any of the preceding claims, wherein the cells are not cancerous.

6. The method of any of the preceding claims, wherein the agent is selected from the group consisting of bacteria, viruses and parasites.

7. The method of any of the preceding claims, wherein the agent is selected from the group consisting of proteins and lipids.

8. The method of any of the preceding claims, wherein the cells are infected with a disease-causing organism.

9. The method of any of the preceding claims, wherein the disease-causing organism is selected from the group consisting of bacteria, viruses and parasites.

10. The method of any of the preceding claims, wherein the cells are blood cells.

11. The method of any of the preceding claims, wherein the cells red blood cells.

12. The method of any of the preceding claims, wherein the cells are white blood cells.

13. The method of any of the preceding claims, further comprising testing the cells, to determine ultrasound frequency and power to selectively kill or induce apoptosis in the cells.

14. The method of any of the preceding claims, wherein the testing comprises testing cells taken from the patient.

15. The method of any of the preceding claims, wherein exposing the cells comprises exposing the cells to a plurality of ultrasound treatments.

16. The method of any of the preceding claims, wherein the patient is infected with a disease-causing organism.

17. The method of any of the preceding claims, wherein the disease-causing organism is selected from the group consisting of bacteria, viruses and parasites.

18. The method of any of the preceding claims, wherein the agent is selected from the group consisting of proteins and lipids.

19. The method of any of the preceding claims, wherein the condition is a chronic disease.

20. A method of removing cells from a sample, comprising:

exposing the sample to ultrasound, to selectively kill or induce apoptosis in the cells,

wherein the cells comprise an accumulated agent.

21. The method of any of the preceding claims, wherein the sample is obtained from a patient.