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(57) Abstract

Disclosed is a method for inhibiting the proliferation of a tumor in a mammal. The method involves the steps of (a) isolating a stress protein-peptide complex from tumor cells previously removed from the mammal and (b) administering the isolated stress protein-peptide complex back to the mammal in order to stimulate in the mammal an immune response against the tumor from which the complex was isolated. Stress protein-peptide complexes having particular utility in the practice of the instant invention include the Hsp70-peptide, Hsp90-peptide and gp96-peptide complexes.

IMMUNOTHERAPEUTIC STRESS PROTEIN-PEPTIDE COMPLEXES AGAINST CANCER

Field of the Invention

The application relates generally to the field of cancer therapy, in particular, to the immunotherapy of human cancer.

Background of the Invention

It has been found that inbred mice and rats can be immunized phrophylactically against tumors derived from mice and rats of the same genetic background (Gross (1943) Cancer Res. 3:323-326; Prehn et al. (1957) J. Natl. Cancer Inst. 18:769-778; Klein et al. (1960) Cancer Res. 20:1561-1572; Old et al. (1962) Ann NY Acad. Sci. 101:80-106; for review, see Srivastava et al. (1988) Immunology Today 9:78-83). These studies not only showed that mice vaccinated with inactivated cancer cells become immunized against subsequent challenges of live cancer cells but also demonstrated the existence of tumor-specific antigens.

Further studies revealed that the phenomenon of prophylactically induced immunity is tumor-specific. Although mice can be specifically immunized against the tumor cells that were used to immunize them they still remain sensitive to challenges with other unrelated tumors (Basombrio (1970) Cancer Res. 30:2458-2462, Globerson et al. (1964) J. Natl. Cancer Inst. 32:1229-1243). The demonstration of immunogenicity of cancer cells led to a search for the cancer-derived molecules which elicit resistance to tumor challenges. The

general approach was to fractionate cancer cell-derived proteins and test them individually for their ability to immunize mice against the cancers from which the fractions were prepared (see Srivastava et al. (1988) supra; Old (1981) Cancer Res. 41:361-375). A number of proteins have been identified by this method, however, a large proportion of these proteins are related to a class of proteins known as stress-induced proteins or stress proteins (Lindquist et al. (1988) Annual Rev. Genet. 22:631-677). Because the stress proteins are among the most highly conserved and abundant proteins in nature, they are unlikely candidates for tumor specific antigens. Stress proteins have subsequently been shown to non covalently associate with a variety of peptides thereby to form stress protein-peptide complexes (Gething et al. (1992) Nature 355:33-45; Lindquist et al. (1988) supra; Young (1990) Annu. Rev. Immunol. 8:401-420; Flynn et al. 1991) Nature 353:726-730).

Studies have also shown that stress protein-peptide complexes lose their immunogenicity upon treatment with ATP (Udono et al. (1993) J. Exp. Med. 178:1391-1396). This treatment is known to dissociate the stress-protein peptide complex into its stress protein and peptide components. Considering that there are no differences in the structure of stress proteins derived from normal and tumor cells, and that stress proteins bind a wide spectrum of peptides in an ATP dependent manner it appears that the antigenicity of the stress protein-peptide complex results not from the stress protein per se, but from the peptide associated with the stress protein.

One of the major conceptual difficulties in cancer immunotherapy has been the possibility that human cancers, like cancers of experimental animals, are antigenically distinct. Clearly, there is some recent evidence for existence of common tumor antigens (Kawakami et al. (1992) J. Immunol. 148:638-643; Darrow et al. (1989) J. Immunol. 142:3329-3334), and this augurs well for prospects of cancer immunotherapy. Nonetheless, in light of the overwhelming evidence from experimental and human systems, it is reasonable to assume that at the very least, human tumors would show tremendous antigenic diversity and heterogeneity.

The prospect of identification of the immunogenic antigens of individual tumors from cancer patients (or even of 'only' several different types of immunogenic antigens in case the antigens are shared), is daunting to the extent of being impractical. Conventional cancer therapies typically are based on the isolation and characterization of specific antigenic determinants which then may become the target for subsequent immunotherapies. In addition, although studies have demonstrated that mammals can be immunized prophylactically against tumors derived from mammals of the same genetic background, heretofore it has not been appreciated that a mammal harboring a tumor can be therapeutically immunized with a composition derived from its own tumor as a means of treating a cancer preexisting in the mammal.

Accordingly, it is an object of the instant invention to provide a novel method for therapeutically inhibiting proliferation of tumors in a mammal. The

method described herein does not require the isolation and characterization of specific antigenic determinants, and accordingly provides a more rapid approach for making and using immunogenic compositions effective in inhibiting the proliferation of specific predetermined tumors in mammals.

This and other objects and features of the invention will be apparent from the description and claims which follow.

Summary of the Invention

The observation that stress proteins chaperone the antigenic peptides of the cells from which they are derived provides an approach for readily isolating antigenic peptides for a preselected tumor. Once isolated, the stress protein-peptide complexes are administered back to the animal from which they were derived in order to elicit an immune response against a preexisting tumor. Accordingly, this approach circumvents the necessity of isolating and characterizing specific tumor antigens and enables the artisan to readily prepare immunogenic compositions effective against a preselected tumor.

In its broadest aspect, the invention provides a method for inhibiting proliferation of a preselected tumor in a mammal. The method comprises administering to the mammal undergoing therapy a composition comprising a pharmaceutically acceptable carrier in combination with a stress protein-peptide complex. The complex having been isolated from a tumor cell previously excised from the mammal and characterized in that it is operative to initiate in the mammal an immune response against the tumor cells from which it was derived. The complex subsequently is administered back to the mammal in an amount sufficient to elicit in the mammal an immune response against the tumor cells thereby to inhibit proliferation of any tumor cells still remaining in the mammal.

It is contemplated that this approach may be used in combination with other conventional cancer therapies

which include, for example, surgery, radiation therapy and chemotherapy. For example, following surgical excision of cancerous tissue the artisan, using the principles described herein, may isolate stress protein-peptide complexes from the excised tissue and administer the complex back to the mammal. The complex subsequently induces a specific immune response against any remaining tumor cells that were not excised during surgery. The approach is amenable to cancer therapy when the primary tumor has metastasized to different locations with the body.

The term "tumor" as used herein, is understood to mean any abnormal or uncontrolled growth of cells which may result in the invasion of normal tissues. It is contemplated also that the term embraces abnormal or uncontrolled cell growths that have metastasized, i.e., abnormal cells that have spread from a primary location in the body (i.e., primary tumor) to a secondary location spatially removed from the primary tumor.

The term "stress protein" as used herein, is understood to mean any cellular protein which satisfies the following criteria. It is a protein whose intracellular concentration increases when a cell is exposed to stressful stimuli, is capable of binding other proteins or peptides, and is capable of releasing the bound proteins or peptides in the presence of adenosine triphosphate (ATP) and/or low pH. Stressful stimuli include, but are not limited to, heat shock, nutrient deprivation, metabolic disruption, oxygen radicals, and infection with intracellular pathogens.

The first stress proteins to be identified were the heat shock proteins (Hsp's). As their name suggests, Hsp's typically are induced by a cell in response to heat shock. Three major families of mammalian Hsp's have been identified to date and include Hsp60, Hsp70 and Hsp90. The numbers reflect the approximate molecular weight of the stress proteins in kilodaltons. The members of each of the families are highly conserved, see for example, Bardwell et al. (1984) Proc. Natl. Acad. Sci. 81:848-852; Hickey et al. (1989) Mol. Cell Biol. 9:2615-2626; Jindal (1989) Mol. Cell. Biol. 9:2279-2283, the disclosures of which are incorporated herein by reference. Members of the mammalian Hsp90 family identified to date include cytosolic Hsp90 (also known as Hsp83) and the endoplasmic reticulum counterparts Hsp90 (also known as Hsp83), Hsp87, Grp94 (also known as ERp99) and gp96. See for example, Gething et al. (1992) Nature 355:33-45 the disclosure of which is incorporated herein by reference. Members of the Hsp70 family identified to date include: cytosolic Hsp70 (also known as p73) and Hsc70 (also known as p72); the endoplasmic reticulum counterpart BiP (also known as Grp78); and the mitochondrial counterpart Hsp 70 (also known as Grp75), Gething et al. (1992) supra. To date, members of the mammalian Hsp60 family have only been identified in the mitochondria, Gething et al. (1992) supra.

In addition, it has been discovered that the Hsp-60, Hsp-70 and Hsp-90 families are composed of proteins related to the stress proteins in amino acid sequence, for example, having greater than 35% amino acid identity, but whose expression levels are not altered by stressful stimuli. Accordingly, it is contemplated

that the definition of stress protein, as used herein, embraces other proteins, muteins, analogs, and variants thereof having at least 35% to 55%, preferably 55% to 75%, and most preferably 75% to 85% amino acid identity with members of the three families whose expression levels in a cell are stimulated in response to stressful stimuli.

The term "peptide", as used herein, is understood to mean any amino acid sequence isolated from a mammalian tumor cell in the form of a stress protein-peptide complex.

The term "immunogenic stress protein-peptide complex", as used herein, is understood to mean any complex which can be isolated from a mammalian tumor cell and comprises a stress protein non covalently associated with a peptide. The complex is further characterized in that it is operative to induce in the mammal an immune response against the tumor cells from which the complex was derived.

The term "immune response" is understood to mean any cellular process that is produced in the mammal following stimulation with an antigen and is directed toward the elimination of the antigen from the mammal. The immune response typically is mediated by one or more populations of cells characterized as being lymphocytic and/or phagocytic in nature.

In a more specific aspect of the invention, the stress protein in the stress protein-peptide complex is selected from the group consisting of Hsp70, Hsp90 and gp96. Stress protein-peptide complexes which include

Hsp70-peptide, Hsp90-peptide and gp96-peptide complexes may be isolated simultaneously from a batch of tumor cells excised from a mammal. During immunotherapy it is contemplated that one or more of the aforementioned complexes may be administered to the mammal in order to stimulate the optimal immune response against the tumor.

It is contemplated that the method described herein is particularly useful in the treatment of human cancer. However, it is contemplated that the methods described herein likewise will be useful in immunotherapy of cancers in other mammals, for example, farm animals (i.e., cattle, horses, goats, sheep and pigs) and household pets (i.e., cats and dogs).

In another aspect of the invention, it is contemplated that the immune response is effected by means of a T cell cascade, and more specifically by means of a cytotoxic T cell cascade. The term "cytotoxic T cell", as used herein, is understood to mean any T lymphocyte expressing the cell surface glycoprotein marker CD8 that is capable of targeting and lysing a target cell which bears a class I histocompatibility complex on its cell surface and is infected with an intracellular pathogen.

In another aspect of the invention, the stress protein-peptide complexes may be administered to the mammal in combination with a therapeutically active amount of a cytokine. As used herein, the term "cytokine" is meant to mean any secreted polypeptide that influences the function of other cells mediating an immune response. Accordingly, it is contemplated

that the complex can be coadministered with a cytokine to enhance the immune response directed against the tumor. Preferred cytokines include, but are not limited to, interleukin-1α (IL-1α), interleukin-1β (IL-1β), interleukin-2 (IL-2), interleukin-3 (IL-3), interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), interleukin-7 (IL-7), interleukin-8 (IL-8), interleukin-9 (IL-9), interleukin-10 (IL-10), interleukin-11 (IL-11), interleukin-12 (IL-12), interferon α (IFNα), interferon β (IFNβ), interferon γ (IFNγ), tumor necrosis factor α (TNFα), tumor necrosis factor β (TNFβ), granulocyte colony stimulating factor (G-CSF), granulocyte/macrophage colony stimulating factor (GM-CSF), and transforming growth factor β (TGF-β).

The complex may be administered to a mammal when combined with a conventional pharmaceutically acceptable carrier, adjuvant, or excipient using techniques well known in the art. The dosage and means of administration of the family of stress protein-peptide complexes necessarily will depend upon a variety of factors such as the stability of the complex under physiological conditions, the effectiveness of the complex at eliciting an immune response, the size and distribution of the tumor, and the age, sex and weight of the mammal undergoing therapy.

Typically, the complex should be administered in an amount sufficient to initiate in the mammal an immune response against the tumor from which the complex was derived and in an amount sufficient to inhibit proliferation of the tumor cells in the mammal. The amount of stress protein-peptide complex administered

preferably is in the range of about 1-1000 micrograms of complex/kg body weight of the mammal/administration, and most preferably about 100-250 micrograms of complex/kg body weight of the mammal/administration. It is contemplated that typical dose will be in the range of about 5 to about 20mg for a human subject weighing about 75 kg. In addition, it is contemplated that the strength of the immune response may be enhanced by repeatedly administering the complex to the individual. The mammal preferably receives at least two doses of the stress protein-peptide complex at weekly intervals. If necessary, the immune response may be boosted at a later date by subsequent administration of the complex. It is contemplated, however, that the optimal dosage and immunization regimen may be found by routine experimentation by one skilled in the art.

Detailed Description.

The invention is based on the observation that stress protein-peptide complexes chaperone antigenic peptides of the cells from which they are derived. Conventional cancer therapies are based upon the isolation an characterization of tumor specific antigens which then become the target for a specific therapeutic regime. Because of the antigenic diversity of mammalian cancers the isolation and characterization of specific tumor antigens for each specific tumor can be impractical. The instant invention thus provides an alternative approach to cancer immunotherapy by obviating the necessity of isolating and characterizing tumor specific antigens for each tumor being treated.

The invention described herein provides a method for inhibiting proliferation of a preselected tumor in a mammal. The method comprises isolating or obtaining tumor cells from the mammal undergoing therapy. This is accomplished readily using conventional surgical procedures well known in the art. Typically, tumor cells are excised from the mammal during routine surgical recision of the tumor. The method then involves isolating stress protein-peptide complexes from the excised tumor cells. This is accomplished using any one of the isolation procedures described in detail herein below. The stress protein-peptide complexes are characterized in that when they are administered back to the mammal they are capable of initiating a specific immune response against the same type of tumor cells that they were derived from. Finally, the method comprises the step of administering back to the mammal the isolated stress protein-peptide complex in an amount sufficient to elicit in the mammal an immune response against the tumor cells thereby

inhibiting proliferation of any tumor cells remaining in the mammal.

It is contemplated that this approach may be used in combination with one or more conventional cancer therapies which include, for example, surgery, radiation therapy and chemotherapy. For example, following surgical excision of cancerous tissue the artisan, using the principles described herein, may isolate stress protein-peptide complexes from the excised tissue and administer the complex back to the mammal. The complex then induces in the mammal a specific immune response against any tumor cells that were not removed during surgery. Alternatively, the method described herein provides a novel approach for treating cancer when the primary tumor has metastasized to multiple locations with the body. For example, when the cancer has metastasized, making surgical intervention impractical, a stress protein-peptide complex may be used either alone or in combination with another standard chemotherapeutic agent in the treatment of the cancer.

It is contemplated that the invention has particular utility in the immunotherapy of human cancer, however, it is appreciated that the methodologies described herein may be applied to the treatment of cancers occurring in, for example, farm animals (i.e., cattle, horses, sheep, goats and pigs) and household pets (i.e., cats and dogs).

The main advantage this approach has over conventional methodologies is that it is not necessary to isolate and characterize the tumor specific antigen for each tumor. Once the stress protein-peptide complex has been isolated it is simply administered back the mammal without further characterization. Since the procedures for isolating the immunogenic complexes are routine and well known in the art, the artisan may rapidly and routinely prepare a specific immunogenic composition "tailor-made" for each individual being treated.

Another advantage of the instant method over previous methodologies is that the administration of purified stress protein-peptide complexes back to the individual from which they were derived eliminates the risk of inoculating the mammal undergoing therapy with potentially transforming agents (i.e., transforming DNA) and/or immunosuppressive agents which can be an issue when the complex is present in a biochemically undefined tumor or tumor extract. In addition, stress protein-peptide complexes can induce significant tumor immunity in the absence of adjuvants. Accordingly, while adjuvants may further enhance the immunotherapeutic properties of the complex, their availability is not a pre-condition for inducing a significant immune response.

It is contemplated that this method can be used in the treatment of a variety of tumors, for example, tumors that are mesenchymal in origin (sarcomas) i.e., fibrosarcomas; myxosarcomas; liposarcomas; chondrosarcomas; osteogenic sarcomas; angiosarcomas; endotheliosarcomas; lymphangiosarcomas;

synoviosarcomas; mesotheliosarcomas; Ewing's tumors; myelogenous leukemias; monocytic leukemias; malignant lymphomas; lymphocytic leukemias; plasmacytomas; leiomyosarcomas and rhabdomyosarcoma.

In addition, it is contemplated that this method can be used in the treatment of tumors that are epithelial in origin (carcinomas) i.e., squamous cell or epidermal carcinomas; basal cell carcinomas; sweat gland carcinomas; sebaceous gland carcinomas; adenocarcinomas; papillary carcinomas; papillary adenocarcinomas; cystadenocarcinomas; medullary carcinomas; undifferentiated carcinomas (simplex carcinomas); bronchogenic carcinomas; bronchial carcinomas; melanocarcinomas; renal cell carcinomas; hepatocellular carcinomas; bile duct carcinomas; papillary carcinomas; transitional cell carcinomas; squamous cell carcinomas; choriocarcinomas; seminomas; embryonal carcinomas malignant teratomas and teratocarcinomas. Generic methodologies useful in the preparation of compositions effective at inducing an immune response against these tumors are discussed in detail herein below.

Although not wishing to be bound by theory, it is contemplated that the stress protein-peptide complexes stimulate an immune response against the tumor cells from which they are derived by means of a T cell cascade. Previous experiments have demonstrated that mice immunized prophylactically with stress protein-peptide preparations derived from a tumor originating in the same strain of mouse or rat develop immunological resistance to the tumor from which it was isolated. The mice, however, fail to develop immunity

against antigenically distinct tumors. Furthermore, stress protein-peptide complexes derived from normal tissues do not elicit resistance to any tumors tested. See for example, Srivastava et al. (1984) Int. J. Cancer 33:417; Srivastava et al. (1986) Proc. Natl. Acad. Sci. USA 83:3407; Palladino et al. (1987) Cancer Res. 47:5074; Feldweg et al. (1993) J. Cell Biochem. Suppl. 17D:108 (Abst.); Udono et al. (1993) J. Cell. Biochem. Suppl. 17D:113 and Udono (1993) J. Exp. Med.178:1391-1396, the disclosures of which are incorporated herein by reference. Recently, it has been established prophylactic immunity typically is mediated by means of a T cell cascade, more specifically by means of a cytotoxic T cell cascade. See for example, Blachere et al. (1993) J. Immunother. 14:352-356, the disclosure of which is incorporated by reference herein. Accordingly, it is contemplated that the stress-protein complexes may also mediate their effect therapeutically by a similar mechanism; specifically, via a cytotoxic T cell cascade.

It is contemplated that the stress protein-peptide complexes typically will be isolated directly from tumor tissue excised from the mammal being treated. Under certain conditions, however, the amount of tumor tissue available for isolation of the complex may be limiting. Accordingly, it is contemplated that the excised tumor tissue may be proliferated using techniques well known in the art prior to the isolation of the stress protein-peptide complexes. For example, the excised tumor tissue may be proliferated either in vivo, for example, by transfecting a nude mouse with a sample of the tumor tissue, or in vitro, for example, by serially passaging the tumor cells in culture. The

proliferated tumor tissue subsequently can be harvested and used as a starting material for the isolation of the stress protein-peptide complex.

Stress proteins useful in the practice of the instant invention may be defined as any cellular protein that satisfies the following criteria. It is a protein whose intracellular concentration increases when a cell is exposed to a stressful stimuli, is capable of binding other proteins or peptides, and is capable of releasing the bound proteins or peptides in the presence of adenosine triphosphate (ATP) or low pH.

The first stress proteins to be identified were the Hsp's which are synthesized in a cell in response to heat shock. To date, three major families of mammalian Hsp's have been identified and include Hsp60, Hsp70 and Hsp90 where the numbers reflect the approximate molecular weight of the stress proteins in kilodaltons. Many members of these families were found subsequently to be induced in response to other stressful stimuli including, but not limited to, nutrient deprivation, metabolic disruption, oxygen radicals, and infection with intracellular pathogens. See for example: Welch (May 1993) Scientific American 56-64; Young (1990) supra; Craig (1993) Science 260:1902-1903; Gething et al (1992) supra; and Lindquist et al. (1988) supra, the disclosures of which are incorporated herein by reference. It is contemplated that mammalian stress proteins belonging to all three families may be useful in the practice of the instant invention.

The major stress proteins accumulate to very high levels in stressed cells but occur at low to moderate

levels in cells that have not been stressed. For example, the highly inducible mammalian Hsp70 is hardly detectable at normal temperatures but becomes one of the most actively synthesized proteins in the cell upon heat shock (Welch et al. (1985), J. Cell. Biol. 101:1198-1211). In contrast, Hsp90 and Hsp60 proteins are abundant at normal temperatures in most, but not all, mammalian cells and are further induced by heat (Lai et al. (1984), Mol. Cell. Biol. 4:2802-10; van Bergen en Henegouwen et al. (1987), Genes Dev., 1:525-31).

Members of the mammalian Hsp90 family identified to date include cytosolic Hsp90 (also known as Hsp83) and the endoplasmic reticulum counterparts Hsp90 (also known as Hsp83), Hsp87, Grp94 (also known as ERp99) and gp96 (Gething et al. (1992) supra). Members of the Hsp70 family identified to date include: cytosolic Hsp70 (also known as p73) and Hsc70 (also known as p72), the endoplasmic reticulum counterpart BiP (also known as Grp78) and the mitochondrial counterpart Hsp 70 (also known as Grp75), Gething et al. (1992) supra. To date, members of the mammalian Hsp60 family have only been identified in the mitochondria, Gething et al. (1992) supra.

Stress proteins are among the most highly conserved proteins in existence. For example, DnaK, the Hsp70 from E. coli has about 50% amino acid sequence identity with Hsp70 proteins from eukaryotes (Bardwell et al. (1984) Proc. Natl. Acad. Sci. 81:848-852). The Hsp60 and Hsp90 families similarly exhibit high levels of intrafamilial conservation (Hickey et al. (1989) Mol. Cell Biol. 9:2615-2626; Jindal (1989) Mol. Cell. Biol.

9:2279-2283). In addition, it has been discovered that the Hsp60, Hsp70 and Hsp90 families are composed of proteins that are related to the stress proteins in sequence, for example, having greater than 35% amino acid identity, but whose expression levels are not altered by stress. Therefore it is contemplated that the definition of stress protein, as used herein, embraces other proteins, muteins, analogs, and variants thereof having at least 35% to 55%, preferably 55% to 75%, and most preferably 75% to 85% amino acid identity with members of the three families whose expression levels in a cell are enhanced in response to a stressful stimulus.

The immunogenic stress protein-peptide complexes of the invention may include any complex containing a stress protein non covalently associated with a peptide that is capable of inducing an immune response in a mammal. Preferred complexes include, but are not limited to, Hsp70-peptide, Hsp90-peptide and gp96-peptide complexes. For example, the mammalian stress protein gp96 which is the endoplasmic reticulum counterpart of the cytosolic Hsp90 may be used in the practice of the instant invention.

Typical procedures for isolating stress protein-peptide complexes useful in the practice of the instant invention are set forth in detail below.

Purification of Hsp70-peptide complexes.

The purification of Hsp70-peptide complexes has been described previously, see for example, Udono et al. (1993) supra.

Initially, tumor cells are suspended in 3 volumes of 1X Lysis buffer consisting of 5mM sodium phosphate buffer (pH7), 150mM NaCl, 2mM $CaCl_2$, 2mM $MgCl_2$ and 1mM phenyl methyl sulfonyl fluoride (PMSF). Then, the pellet is sonicated, on ice, until >99% cells are lysed as determined by microscopic examination. As an alternative to sonication, the cells may be lysed by mechanical shearing and in this approach the cells typically are resuspended in 30mM sodium bicarbonate pH 7.5, 1mM PMSF, incubated on ice for 20 min and then homogenized in a dounce homogenizer until >95% cells are lysed.

Then the lysate is centrifuged at 1000g for 10 minutes to remove unbroken cells, nuclei and other cellular debris. The resulting supernatant is recentrifuged at 100,000g for 90 minutes, the supernatant harvested and then mixed with Con A Sepharose equilibrated with phosphate buffered saline (PBS) containing 2mM Ca^{2+} and 2mM Mg^{2+}. When the cells are lysed by mechanical shearing the supernatant is diluted with an equal volume of 2X Lysis buffer prior to mixing with Con A Sepharose. The supernatant is then allowed to bind to the Con A Sepharose for 2-3 hours at 4°C. The material that fails to bind is harvested and dialyzed for 36 hours (three times, 100 volumes each time) against 10mM Tris-Acetate pH 7.5, 0.1 mM EDTA, 10mM NaCl, 1mM PMSF. Then the dialyzate is centrifuged at 17,000 rpm (Sorvall SS34 rotor) for 20 min. Then the resulting supernatant is harvested and applied to a Mono Q FPLC column equilibrated in 20mM Tris-Acetate pH 7.5, 20 mM NaCl, 0.1mM EDTA and 15mM 2-mercaptoethanol. The column is then developed with a 20 mM to 500 mM NaCl gradient and the eluted

fractions fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and characterized by immunoblotting using an appropriate anti-Hsp70 antibody (such as from clone N27F3-4, from StressGen).

Fractions strongly immunoreactive with the anti-Hsp70 antibody are pooled and the Hsp70-peptide complexes precipitated with ammonium sulfate; specifically with a 50%-70% ammonium sulfate cut. The resulting precipitate is then harvested by centrifugation at 17,000 rpm (SS34 Sorvall rotor) and washed with 70% ammonium sulfate. The washed precipitate is then solubilized and any residual ammonium sulfate removed by gel filtration on a Sephadex^{R} G25 column (Pharmacia).

The Hsp70-peptide complex can be purified to apparent homogeneity using this method. Typically 1mg of Hsp70-peptide complex can be purified from 1g of cells/tissue.

Purification of Hsp90-peptide complexes.

Initially, tumor cells are suspended in 3 volumes of 1X Lysis buffer consisting of 5mM sodium phosphate buffer (pH7), 150mM NaCl, 2mM $CaCl_2$, 2mM $MgCl_2$ and 1mM phenyl methyl sulfonyl fluoride (PMSF). Then, the pellet is sonicated, on ice, until >99% cells are lysed as determined by microscopic examination. As an alternative to sonication, the cells may be lysed by mechanical shearing and in this approach the cells typically are resuspended in 30mM sodium bicarbonate pH 7.5, 1mM PMSF, incubated on ice for 20 min and then

homogenized in a dounce homogenizer until >95% cells are lysed.

Then the lysate is centrifuged at 1000g for 10 minutes to remove unbroken cells, nuclei and other cellular debris. The resulting supernatant is recentrifuged at 100,000g for 90 minutes, the supernatant harvested and then mixed with Con A Sepharose equilibrated with PBS containing 2mM Ca^{2+} and 2mM Mg^{2+}. When the cells are lysed by mechanical shearing the supernatant is diluted with an equal volume of 2X Lysis buffer prior to mixing with Con A Sepharose. The supernatant is then allowed to bind to the Con A Sepharose for 2-3 hours at 4°C. The material that fails to bind is harvested and dialyzed for 36 hours (three times, 100 volumes each time) against 10mM Tris-Acetate pH 7.5, 0.1 mM EDTA, 10mM NaCl, 1mM PMSF. Then the dialyzate is centrifuged at 17,000 rpm (Sorvall SS34 rotor) for 20 min. Then the resulting supernatant is harvested and applied to a Mono Q FPLC column equilibrated equilibrated with lysis buffer. The proteins are then eluted with a a salt gradient of 200mM to 600mM NaCl.

The eluted fractions are fractionated by SDS-PAGE and fractions containing the Hsp90-peptide complexes identified by immunoblotting using a anti-Hsp90 antibody such as 3G3 (Affinity Bioreagents). Hsp90-peptide complexes can be purified to apparent homogeneity using this procedure. Typically, 150-200 μg of Hsp90-peptide complex can be purified from 1g of cells/tissue.

Purification of gp96-peptide complexes.

Initially, tumor cells are suspended in 3 volumes of 1X Lysis buffer consisting of 5mM sodium phosphate buffer (pH7), 150mM NaCl, 2mM $CaCl_2$, 2mM $MgCl_2$ and 1mM phenyl methyl sulfonyl fluoride (PMSF). Then, the pellet is sonicated, on ice, until >99% cells are lysed as determined by microscopic examination. As an alternative to sonication, the cells may be lysed by mechanical shearing and in this approach the cells typically are resuspended in 30mM sodium bicarbonate pH 7.5, 1mM PMSF, incubated on ice for 20 min and then homogenized in a dounce homogenizer until >95% cells are lysed.

Then the lysate is centrifuged at 1000g for 10 minutes to remove unbroken cells, nuclei and other cellular debris. The resulting supernatant is recentrifuged at 100,000g for 90 minutes, the supernatant harvested and mixed with Con A Sepharose slurry equilibrated with PBS containing 2mM Ca^{2+} and 2mM Mg^{2+}. When the cells are lysed by mechanical shearing the supernatant is diluted with an equal volume of 2X Lysis buffer prior to mixing with Con A Sepharose. The supernatant is then allowed to bind to the Con A Sepharose for 2-3 hours at 4°C. The slurry is then packed into a column and washed with 1X lysis buffer until the OD_{280} drops to baseline. Then the column is washed with 1/2 column bed volume of 10% α-methyl mannoside (α-MM), the column sealed with parafilm and incubated at 37°C for 15 min. The column is then cooled to room temperature, the parafilm removed from the bottom of the column, and five column volumes of a α-MM is applied to the column. The eluate

is then fractionated and characterized by SDS-PAGE. Typically, the resulting gp96-peptide complex is about 60 to 95% pure depending upon the cell type and the tissue to lysis buffer ratio used.

If further purification is required, the sample can be applied to a Mono Q FPLC column equilibrated with a buffer containing 5mM sodium phosphate, pH7. The proteins are then eluted from the column with a 0-1M NaCl gradient. The gp96 fraction elutes between 400mM and 550mM NaCl.

As an alternative procedure, the gp96 fraction isolated from the 100,000g pellet can be resuspended in 5 volumes of PBS containing 1% sodium deoxycholate (without Ca^{2+} and Mg^{2+}) and incubated on ice for 1 h. The resulting suspension is centrifuged for 30 min at 20,000g and the resulting supernatant harvested and dialyzed against several changes of PBS (without Ca^{2+} and Mg^{2+}) to remove the detergent. The resulting dialysate is centrifuged for 90 min at 100,000g and the supernatant purified further. Then calcium and magnesium are both added to the supernatant to give final concentrations of 2mM. Then the sample is applied to a Mono Q HPLC column equilibrated with a buffer containing 5mM sodium phosphate, pH7 and the proteins eluted with a 0-1M NaCl gradient. The gp96 fraction elutes between 400mM and 550mM NaCl.

The gp96-peptide complexes can be purified to apparent homogeneity using this procedure. Typically about 10-20 μg of gp96 can be isolated from 1g cells/tissue using this method.

Formulation and Administration of the Complexes.

Once stress protein-peptide complexes have been purified from the excised tumor they are administered back to the mammal undergoing therapy in order to stimulate in the mammal an immune response against tumor cells from which the complex was derived. The stress protein-peptide complexes of the invention may either be stored or prepared for administration by mixing with physiologically acceptable carriers, excipients, or stabilizers. These materials should be non-toxic to the intended recipient at dosages and concentrations employed.

When the complex is water soluble it may be formulated in an appropriate buffer, for example PBS (5mM sodium phosphate, 150 mM NaCl, pH7.1) or other physiologically compatible solutions. Alternatively, if the resulting complex has poor solubility in aqueous solvents then it may be formulated with a non-ionic surfactant such as Tween, or polyethylene glycol.

Useful solutions for oral or parenteral administration may be prepared by any of the methods well known in the pharmaceutical art, described, for example, in Remington's Pharmaceutical Sciences, (Gennaro, A., ed.), Mack Pub., 1990. Formulations may include, for example, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, hydrogenated naphthalenes, and the like. Formulations for direct administration, in particular, may include glycerol and other compositions of high viscosity. Biocompatible, preferably bioresorbable polymers, including, for example, hyaluronic acid, collagen,

tricalcium phosphate, polybutyrate, polylactide, polyglycolide and lactide/glycolide copolymers, may be useful excipients to control the release of the stress protein-peptide complexes in vivo.

Formulations for inhalation may contain as excipients, for example, lactose. Aqueous solutions may contain, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate. Oily solutions may be useful administration in the form of nasal drops. Gels may be applied topically intranasally.

The compounds provided herein can be formulated into pharmaceutical compositions by admixture with pharmaceutically acceptable nontoxic excipients and carriers. In addition the formulations may optionally contain one or more adjuvants. Preferred adjuvants include, but are not limited to, pluronic tri-block copolymers, muramyl dipeptide and its derivatives, detoxified endotoxin, saponin and its derivatives such as QS-21 and liposomes. The present invention further envisages sustained release formulations in which the complex is released over an extended period of time.

The mode of administration of the family of stress protein-peptide complexes prepared in accordance with the invention will necessarily depend upon the stability of the complex under physiological conditions, and the size and distribution of the tumor within the mammal being treated. The preferred dosage of complex to be administered also is likely to depend on such variables as the size and distribution of the tumor, the age, sex and weight of the intended

recipient, the overall health status of the particular recipient, the relative biological efficacy of the complex, the formulation for the complex, the presence and types of excipients in the formulation, and the route of administration.

In general terms, the compounds of this invention may be provided in an aqueous physiological buffer solution containing about 0.001 to 10% w/v compound for parenteral administration. Preferred dosages range from about 1 to about 1000 micrograms of complex/kg body weight of recipient/administration and most preferably range from about 100 to about 250 micrograms of complex/kg body weight of recipient/administration. In particular, it is contemplated that a typical dose will range from about 5mg to about 20mg for a human subject weighing about 75kg. These quantities, however, may vary according to the adjuvant coadministered with the complex.

The complex preferably comprises part of an aqueous solution which may be administered using standard procedures, for example, intravenously, subcutaneously, intramuscularly, intraorbitally, ophthalmically, intraventricularly, intracranially, intracapsularly, intraspinally, intracisternally, intraperitoneally, buccal, rectally, vaginally, intranasally or by aerosol administration. The aqueous solution preferably is physiologically acceptable so that in addition to delivery of the desired complex to the mammal, the solution does not otherwise adversely affect the mammal's electrolyte and/or volume balance. The aqueous medium for the complex thus may comprise normal physiologic saline (0.9% NaCl, 0.15M), pH 7-7.4 or

other pharmaceutically acceptable salts thereof.

Preferably the recipient should be vaccinated three times at two week intervals. If necessary, the responses may be boosted at a later date by subsequent administration of the complex. It is contemplated that the optimal dosage and vaccination schedule may be determined empirically for each stress protein-peptide complex using techniques well known in the art.

Various cytokines, antibiotics, and other bioactive agents also may be coadministered with the stress protein-peptide complexes. For example, various known cytokines, i.e., interleukin-1α (IL-1α), interleukin-1β (IL-1β), interleukin-2 (IL-2), interleukin-3 (IL-3), interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), interleukin-7 (IL-7), interleukin-8 (IL-8), interleukin-9 (IL-9), interleukin-10 (IL-10), interleukin-11 (IL-11), interleukin-12 (IL-12), interferon α (IFNα), interferon β (IFNβ), interferon γ (IFNγ), tumor necrosis factor α (TNFα), tumor necrosis factor β (TNFβ), granulocyte colony stimulating factor (G-CSF), granulocyte/macrophage colony stimulating factor (GM-CSF), and transforming growth factor β (TGF-β) may be coadministered with the complexes in order to maximize the physiological response. However, it is anticipated that other but as yet undiscovered cytokines may be effective in the invention. In addition, conventional antibiotics may be coadministered with the stress protein-peptide complex. The choice of suitable antibiotics will however be dependent upon the disease in question.

Example I

In this example, C57BL/6 and C3H mice approximately 100 g in weight, are purchased from Jackson Laboratories, Bar Harbor, Me. Malignant tumor cells are then injected subcutaneously into mice in order to induce experimental tumors in the mice. Specifically, malignant spindle cell carcinoma 6139 cells are injected subcutaneously into the C3H mice, malignant mouse Lewis lung carcinoma cells are injected subcutaneously into C57BL/6 mice and malignant mouse B16 melanoma cells are injected subcutaneously into C57BL/6 mice.

When the tumors have grown to a size such that they are both visible and palpable, a sample of the tumor tissue is excised. As a control, normal non malignant tissue is excised from some mice bearing the experimental tumors.

Then gp96-peptide, Hsp90-peptide and Hsp70-peptide complexes are isolated from both the excised normal and tumor derived tissues using the methods described hereinabove. Once isolated, the complexes are combined with PBS and administered back to the mice from which the complexes were derived. Usually 6 mice are tested in each experiment. The experiments are performed using the schedule set forth below:

Experiment	Composition administered back to mice
1	gp96-peptide
2	Hsp70-peptide
3	Hsp90-peptide
4	gp96-peptide and Hsp70-peptide
5	gp96-peptide and Hsp90-peptide
6	Hsp70-peptide and Hsp90-peptide
7	Hsp70-peptide, Hsp90-peptide and gp96-peptide
8	buffer alone

In one series of experiments the complexes are isolated from tumor cells whereas in a second series the complexes are isolated from normal cells. The mice are inoculated three times at weekly intervals with 20 micrograms (total weight) of the preselected complex(es). During therapy, the size of each tumor is measured daily. After 4 weeks the mice are sacrificed and the development of the tumor examined histologically. In addition, the sacrificed mice are examined for the presence or absence of metastasis.

It is expected that the tumors in mice treated with complexes derived from normal tissue will continue to grow and metastasize. In contrast, it is expected that the tumors in the mice treated with the complexes derived from the tumor tissue will be exhibit slower growth than the tumors in the control animals, and in some cases, it is expected that the tumor mass may get smaller and the tumor exhibit remission during therapy.

Other Embodiments

The invention may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The present embodiments are therefore to be considered in all respects as illustrative and not restrictive, the scope of the invention being indicated by the appended claims rather than by the foregoing description, and all changes which come within the meaning and range of equivalency of the claims are therefore intended to be embraced therein.

Throughout the description and claims of this specification, the word "comprise" and variations of the word, such as "comprising" and "comprises", is not intended to exclude other additives, components, integers or steps.

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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method for treating a mammal having a tumor sensitive to treatment with a mammalian stress protein-peptide complex comprising administering to the mammal a composition comprising:

(a) an amount of a purified immunogenic mammalian stress protein-peptide complex isolated from a tumor cell sufficient to elicit an immune response against the tumor, wherein the peptide is noncovalently associated with the stress protein; and

(b) a pharmaceutically acceptable carrier.

2. The method of claim 1 wherein the tumor cell is from the mammal.

3. The method of claim 1 wherein the mammal is a human.

4. The method of claim 3 wherein the tumor cell is from a human tumor cell.

5. The method of claim 2 wherein the mammal is a human.

6. A method for treating a mammal having a tumor sensitive to treatment with a mammalian stress protein-peptide complex comprising:

(a) isolating an immunogenic mammalian stress protein-peptide complex from a cell of a tumor, wherein the peptide is noncovalently associated with the stress protein; and

(b) administering a composition comprising an amount of the isolated complex sufficient to elicit an immune response against the tumor, and a pharmaceutically acceptable carrier.

7. The method of claim 6 wherein the tumor cell is from the mammal.

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8. The method of claim 6 wherein the mammal is a human.

9. The method of claim 8 wherein the tumor cell is a human tumor cell.

10. The method of claim 7 wherein the mammal is a human.

11. A method for eliciting in a human an immune response against a tumor comprising administering to the human a composition comprising:

(a) an amount of a purified immunogenic mammalian stress protein-peptide complex isolated from a cell derived from a tumor sufficient to elicit an immune response against the tumor, wherein the peptide is noncovalently associated with the stress protein; and

(b) a pharmaceutically acceptable carrier.

12. The method of claim 1 wherein the stress protein in the complex is a Hsp70, a Hsp90 or a gp96.

13. The method of claim 2 wherein the stress protein in the complex is a Hsp70, a Hsp90 or a gp96.

14. The method of claim 3 wherein the stress protein in the complex is a Hsp70, a Hsp90 or a gp96.

15. The method of claim 4 wherein the stress protein in the complex is a Hsp70, a Hsp90 or a gp96.

16. The method of claim 5 wherein the stress protein in the complex is a Hsp70, a Hsp90 or a gp96.

17. The method of claim 6 wherein the stress protein in the complex is a Hsp70, a Hsp90 or a gp96.

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18. The method of claim 7 wherein the stress protein in the complex is a Hsp70, a Hsp90 or a gp96.

19. The method of claim 8 wherein the stress protein in the complex is a Hsp70, a Hsp90 or a gp96.

20. The method of claim 11 wherein the stress protein in the complex is a Hsp70, a Hsp90 or a gp96.

21. The method of claim 1, 5, 6 or 10 wherein the immune response is mediated by cytotoxic T cells.

22. The method of claim 1, 3, 5, 6, 8 or 10 wherein the tumor has metastasized in said mammal.

23. The method of claim 1, 3, 5, 6, 8 or 10 wherein the tumor is a melanocarcinoma.

24. The method of claim 1, 3, 5, 6, 8 or 10 wherein the tumor is a hepatocellular carcinoma.

25. The method of claim 1, 3, 5, 6, 8 or 10 wherein the tumor is a renal cell carcinoma.

26. The method of claim 1, 3, 5, 6, 8 or 10 wherein the composition comprises a combination of a Hsp70-peptide complex, a Hsp90-peptide complex, and a gp96-peptide complex.

27. The method of claim 11 wherein said administering step is carried out to immunize said human prophylactically against said tumor.

28. The method of claim 11 wherein the tumor is a human tumor.

29. The method of claim 11 or 12 wherein the tumor is a melanocarcinoma.

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30. The method of claim 11 or 12 wherein the tumor is a hepatocellular carcinoma.

31. The method of claim 11 or 12 wherein the tumor is a renal cell carcinoma.

32. The method of claim 11 wherein the composition comprises a combination of a Hsp70-peptide complex, a Hsp90-peptide complex, and a gp96-peptide complex.

33. The method of claim 1 wherein the complex is administered to the mammal in an amount in the range of 1 to 1000 micrograms of complex per kg body weight of mammal per administration.

34. The method of claim 33 wherein the complex is administered to the mammal in an amount in the range of 100 to 250 micrograms of complex per kg body weight of mammal per administration.

35. The method of claim 4 wherein the complex is administered to the mammal in an amount in the range of 1 to 1000 micrograms of complex per kg body weight of mammal per administration.

36. The method of claim 35 wherein the complex is administered to the mammal in an amount in the range of 100 to 250 micrograms of complex per kg body weight of mammal per administration.

37. The method of claim 5 wherein the complex is administered to the mammal in an amount in the range of 1 to 1000 micrograms of complex per kg body weight of mammal per administration.

38. The method of claim 37 wherein the complex is administered to the mammal in an amount in the range of 100

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to 250 micrograms of complex per kg body weight of mammal per administration.

39. The method of claim 6 wherein the complex is administered to the mammal in an amount in the range of 1 to 1000 micrograms of complex per kg body weight of mammal per administration.

40. The method of claim 39 wherein the complex is administered to the mammal in an amount in the range of 100 to 250 micrograms of complex per kg body weight of mammal per administration.

41. The method of claim 7 wherein the complex is administered to the mammal in an amount in the range of 1 to 1000 micrograms of complex per kg body weight of mammal per administration.

42. The method of claim 41 wherein the complex is administered to the mammal in an amount in the range of 100 to 250 micrograms of complex per kg body weight of mammal per administration.

43. The method of claim 1 wherein the complex is administered repeatedly to the mammal.

44. The method of claim 5 wherein the complex is administered repeatedly to the mammal.

45. The method of claim 6 wherein the complex is administered repeatedly to the mammal.

46. The method of claim 1, 3, 5, 6, 8 or 10 wherein the stress protein in the complex is a gp96.

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47. The method of claim 1 wherein the composition is administered in combination with a cytokine.

48. The method of claim 5 wherein the composition is administered in combination with a cytokine.

49. The method of claim 6 wherein the composition is administered in combination with a cytokine.

50. A composition comprising:

(a) an amount effective for treatment of cancer of purified human stress protein-peptide complex isolated from human tumor tissue excised from a human, wherein the peptide is noncovalently associated with the stress protein; and

(b) a pharmaceutically acceptable carrier.

51. The composition of claim 50 wherein the stress protein is gp96.

52. A composition comprising:

(a) an amount effective for the treatment of a cancer of purified human stress protein-peptide complex isolated from a human tumor cell, wherein the peptide is noncovalently associated with the stress protein, and wherein the stress protein is a homogenous Hsp90; as analyzed by sodium dodecyl sulfate-polyacrylanide gel electrophoresis; and

(b) a pharmaceutically acceptable carrier.

53. A composition comprising:

(a) an amount effective for treatment of a cancer of purified human stress protein-peptide complex isolated from a human tumor cell, wherein the peptide is noncovalently associated with the stress protein, and wherein the stress protein-peptide complex is a combination of a Hsp70-peptide complex, a Hsp90-peptide complex, and a human gp96-peptide complex; and

(b) a pharmaceutically acceptable carrier.

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54. An isolated human stress protein-peptide complex isolated from a human tumor cell, wherein the peptide is noncovalently associated with the stress protein, and wherein the stress protein is human gp96.

55. An isolated human stress protein-peptide complex isolated from a human tumor cell, wherein the peptide is noncovalently associated with the stress protein, and wherein the stress protein is a homogenous Hsp90 as analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

56. An isolated human stress protein-peptide complex isolated from a human tumor cell, which is a combination of a Hsp70-peptide complex, a Hsp90-peptide complex, a Hsp90-peptide complex, and a human gp96-peptide complex; and wherein the peptide is noncovalently associated with the stress protein.

57. A composition comprising:

(a) an amount effective for treatment of a cancer of purified human stress protein-peptide complex isolated from a human tumor cell, wherein the peptide is noncovalently associated with the stress protein, and wherein the stress protein is human gp96; and

(b) a pharmaceutically acceptable carrier.

58. A method according to any one of claims 1, 6 or 11 substantially as hereinbefore defined with reference to any of the examples.

59. A composition according to any one of claims 50, 52, 53 or 57 substantially as hereinbefore described.

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