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(54) Title: COMPOSITIONS AND METHODS FOR REGULATING THE CELL CYCLE

(57) Abstract: The present invention relates, generally, to compositions and methods for regulating the cell cycle, in particular apoptosis. The invention relates more specifically to compositions and methods using a Ki gene or polypeptide to regulate the cell cycle, in particular apoptosis, in cells, in vitro, ex vivo or in vivo. The invention also relates to compositions, vectors, cells and the like, which can be used to implement the above methods. This invention can be used to protect cells from apoptosis or to mediate or enhance apoptosis in cells, in various physio-pathological conditions.

Compositions and methods for regulating the cell cycle

Field of Invention

The present invention relates, generally, to compositions and methods for regulating the cell cycle, in particular apoptosis. The invention relates more specifically to compositions and methods using a Ki gene or polypeptide to regulate the cell cycle, in particular apoptosis, in cells, in vitro, ex vivo or in vivo. The invention also relates to compositions, vectors, cells and the like, which can be used to implement the above methods. This invention can be used to protect cells from apoptosis or to mediate or enhance apoptosis in cells, in various physio-pathological conditions.

Background of invention

Homeostasis is achieved through a balance of cell growth and cell death. Many recent studies have been conducted to determine key events which lead to cell death or cell survival (for review see (Best, Hasdai et al. 1999)). The withdrawal of growth factors in vitro (Batistatou and Greene 1993; Wang and Walsh 1996) and in vivo (Colombel, Olsson et al. 1992) from differentiated cells provokes cell cycle arrest and consequently apoptosis. Homeobox transcription factors have been shown to be implicated in cell differentiation and migration and deregulation of the expression of some of them may be associated with abnormal cell proliferation. However most of them have been associated with cell proliferation, some exhibit growth suppressive properties. Among them, a homeobox transcription factor gene, referred as to Gax (Growth Arrest Homeobox) was isolated from an adult aorta VSMC cDNA library (Gorski, LePage et al. 1993; LePage, Altomare et al. 1994). In VSMCs, gax expression is rapidly repressed by growth factor stimulation, and upregulated under conditions that promote quiescence (Gorski, LePage et al. 1993). Similarly, expression of gax is rapidly downregulated in rat carotid arteries following an injury that stimulates VSMC proliferation (Weir, Chen et al. 1995). Constitutive gax

expression in vitro blocks both mitogen-stimulated cell cycle activity and mitogen-directed VSMC migration (Smith, Branellec et al. 1997; Witzenbichler, Kureishi et al. 1999). Under conditions of prolonged mitogen activation, forced gax expression leads to p53-independant apoptotic cell death (Perlman, Sata et al. 1998). We recently reported that gax is able to induce genes which play a key role in cell cycle control and apoptosis such as p21 (Smith, Branellec et al. 1997) and bax and bad respectively (Perlman, Sata et al. 1998).

Abnormal proliferation and migration of VSMCs is associated with vascular disorders such as atherosclerosis and restenosis following the balloon angioplasty (Ross 1993; Stary, Chandler et al. 1995). Given the unusual pattern of gax expression, we decided to investigate its potential use for gene therapy of restenosis. We showed that adenovirus-mediated gax overexpression markedly inhibits injury-induced intimal hyperplasia in both rat carotid artery and rabbit iliac artery models of balloon denudation (Maillard, Van Belle et al. 1997; Perlman, Luo et al. 1999). Moreover gax gene transfer leads first to cell cycle arrest and then cell apoptosis in the rat carotid model (Perlman, Luo et al. 1999). Thus gax is an important gene in SMC biology in vitro and in vivo. Owing to gax properties toward SMCs we wondered whether gax overexpression may affect human tumoral cell proliferation.

The inventors have disclosed in patent application n° WO98/17686, that gax overexpression in human tumoral cell lines leads to cell apoptosis. WO98/17686 also describes a gax functional partner isolated by using the two-hybrid screen in the yeast : the Ki antigen. Ki antigen was first identified as an autoantigen in patients with systemic lupus erythematosus (SLE) (Tojo, Kaburaki et al. 1981). It is a 32 kDa protein which exhibits a nuclear localization and the expression of which has been shown to increase during proliferation and in transformed fibroblasts.

However, so far, the relevance of this interaction remains unclear. More generally, the possible role of Ki in the regulation of cell cycle has not been suggested or reported in the literature.

Summary of Invention

The present invention relates, generally, to compositions and methods for regulating the cell cycle, in particular apoptosis, more specifically using a Ki gene or polypeptide, in vitro, ex vivo or in vivo. The present invention stems from the unexpected discovery that Ki antigen, a component of the proteasome (also referred as to PA28γ), exhibits cyto-protective activity. More particularly, the present invention provides the first evidence that Ki antigen is able to control cell cycle (including apoptosis), since Gax-mediated cell killing activity was reduced by Ki overexpression. Furthermore, this application also demonstrates that Ki protective activity is not restricted to Gax since p53-mediated cytotoxicity towards tumor cells was also reduced by Ki. These data unravel a novel role for Ki, a component of the proteasome activator complex, and provides novel strategies to control or modulate cell cycle, including apoptosis, cell death, degeneration, proliferation, and the like.

In this regard, a particular aspect of the present invention resides in a method of inhibiting or reducing apoptosis in a cell, comprising contacting the cell with a Ki polypeptide or gene.

A further aspect of this invention relates to the use of a Ki polypeptide or gene to inhibit or reduce apoptosis in a cell.

In particular variants, the invention relates to methods of inhibiting or reducing p53-mediated apoptosis or GAX-mediated apoptosis.

A further variant of this invention relates to a method of treating a cell to protect said cell from death or degeneration, comprising contacting the cell with a Ki polypeptide or gene.

According to a preferred embodiment, the method of this invention comprises contacting the cell(s), in vivo or ex vivo, with a vector encoding a Ki polypeptide, such as a viral vector or a plasmid.

Furthermore, the Ki gene or polypeptide may be used alone or in combination(s) with other therapeutic or prophylactic treatment(s) or agent(s).

The above methods/uses can be applied to various cell types, either isolated or in tissues, organs or whole organisms, in healthy or pathological condition, such as more particularly smooth muscle cells (in particular cardiomyocytes), muscle cells, fibroblasts, hepatic cells, endothelial cells, nervous cells, etc.

Another aspect of this invention resides in a method of causing or stimulating (or enhancing) apoptosis in a cell, comprising contacting the cell with a compound that inhibits Ki gene expression or activity. This invention also concerns the use of a compound that inhibits Ki gene expression or activity to cause or stimulate (or enhance) apoptosis in a cell.

More preferably, the compound is an antisense nucleic acid molecule that inhibits Ki gene expression, an anti-Ki antibody, or a chemical inhibitor or competitor, including peptides (or nucleic acids encoding the same).

The compound can be used either alone or in combination(s) with additional cytotoxic agent(s) or treatment(s), such as tumor suppressor genes or polypeptides, anti-tumor chemical agents or treatments, etc. The cytotoxic agent may be used before, after or together with the compound that inhibits Ki gene expression or activity.

These embodiments may be applied to various cell populations, such as abnormally proliferating cells, including tumor cells, SMCs, pathological immune cells (T lymphocytes), etc.

In this regard, a further aspect of this invention resides in compositions comprising a compound that inhibits Ki gene expression or activity and a cytotoxic agent, for simultaneous, separate or sequential use.

The invention also relates to compositions comprising a Ki gene or polypeptide and a cyto-protective agent, for simultaneous, separate or sequential use.

A further aspect of this invention comprises a viral vector comprising a Ki gene, more preferably an adenoviral vector or an AAV-derived vector.

Additional aspects of this invention include compositions, such as pharmaceutical compositions, nucleic acid constructs, recombinant cells as well as methods of treating or preventing various pathological conditions where abnormal cell cycle regulation occurs, such as tumors, neuro-degeneration, stenosis, stroke, myocardial disorders (e.g., myocardial ischemia, heart failure), hepatitis, cyrrhosis, etc.

Legend to the Figures

Figure 1: Transfection assay in H1299 tumor cells. 72hrs after transfection using Lipofectamine, H1299 cells viability was assessed by crystal violet.

Figure 2: Transfection assay in MEF fibroblast cells. 48hrs after transfection using Lipofectamine, MEF cells viability was assessed.

Detailed description of the invention

The present invention relates, generally, to compositions and methods for regulating the cell cycle, in particular apoptosis, more specifically using a Ki gene or polypeptide, in vitro,

ex vivo or in vivo.

More particularly, the regulation of cell cycle comprises, within the context of the present invention, a regulation of growth stimulating and/or growth inhibiting genes or polypeptides or pathways in a cell. The invention is more particularly suited to regulate apoptosis (i.e. programmed cell death) in a cell, or related gene pathways. This invention also includes methods of protecting cell from death or degeneration, i.e., methods of inhibiting or reducing cell death or degeneration. The invention can also be used to prolong cell survival.

According to this invention, inhibiting or reducing means either partial or complete suppression, more generally partial suppression. In a typical embodiment, inhibiting cell death designates an inhibition of at least 20%, preferably at least 30% of the cellular death, or a prolonged cell survival time of at least 20%, more preferably at least 30%. Similarly, an inhibition of apoptosis designates an inhibition of at least 20%, preferably at least 30% of the cellular death, or a prolonged cell survival time of at least 20%, more preferably at least 30%.

The present invention is based on the use of a Ki gene or polypeptide. Within the context of the present invention, Ki polypeptide designates the human Ki polypeptide comprising the amino acid sequence SEQ ID NO:2. A Ki polypeptide also includes any variants or homologs of the above sequence, more particularly naturally-occurring variants or homologs, such as polymorphisms, splicing variants, homologs from other species, etc., that have the ability to interact with a GAX polypeptide in vitro, or to reduce Gax-mediated or p53-mediated apoptosis in a cell. Ki polypeptides also include derivatives of the above sequence, comprising one or several amino acid modifications, such as a mutation, a deletion, a substitution and/or an addition of one or several amino acid residues, and having the above-described property(ies). Preferably, a Ki polypeptide has at least 75% amino acid identity, even more preferably at least 90% amino acid identity with sequence SEQ ID

NO:2. A Ki polypeptide may also be modified by addition of a foreign moiety, such as a tag, marker, linker, etc. More generally, Ki polypeptides include any polypeptide encoded by a nucleic acid molecule that hybridises, under stringent conditions, with a nucleic acid encoding the Ki polypeptide of SEQ ID NO:2 and having the above-mentioned property(ies).

A Ki gene designates any nucleic acid molecule encoding a Ki polypeptide as described above, more preferably a Ki polypeptide having the sequence of SEQ ID NO:2. The Ki gene may be a DNA (cDNA, gDNA, synthetic or semi-synthetic DNA, etc.) or a RNA (mRNA, premRNA, synthetic or semi-synthetic RNA, etc.). In a preferred embodiment, a Ki gene comprises the nucleic acid sequence SEQ ID NO:1, and is, more preferably, a cDNA.

As mentioned above, a particular object of the present invention resides in a method of inhibiting or reducing apoptosis in a cell, comprising contacting the cell with a Ki polypeptide or gene.

In particular variants, the invention relates to methods of inhibiting or reducing p53-mediated apoptosis or GAX-mediated apoptosis. Apoptosis has been associated with various pathological conditions and occurs in response to various stimuli. The invention now offers novel and efficient strategies to counteract the effect of apoptosis, such as Gax- or p53-mediated apoptosis.

According to a preferred embodiment, the method of this invention comprises contacting the cell(s), in vivo or ex vivo (or in vitro), with a vector encoding a Ki polypeptide, such as a viral vector or a plasmid. Indeed, it is preferred to deliver a Ki gene to the cells (or tissues, organs, organisms), allowing the gene to be expressed in the cells and to produce its cyto-protective effect.

Preferred vectors are viral vectors such as adenoviral vectors, AAV-based vectors or lentiviral vectors, more specifically replication-defective adenoviral vectors, lentiviral vectors or AAV-based vectors. More generally, any viral vector that can infect target cells such as cardiomyocytes, skeletal myocytes, smooth muscle cells or neuronal cells can be used. Adenoviral vectors may be prepared from various serotypes, such as Ad2 or Ad5, by deleting one or several viral genes (such as E1, E4, E2, E3 and/or Iva2, etc.), and by inserting the Ki gene in said defective vector. The viruses may be produced in any suitable packaging cell, according to techniques known in the art. AAV-based vectors may be prepared from various serotypes, by deleting one or several viral genes (such as Rep and/or Cap), and by inserting the Ki gene in said defective vector. The viruses may be produced in any suitable packaging cell, according to techniques known in the art. Specific Adenoviral vectors have been described for instance in WO00/12738.

Preferred viral vectors are:

- replication-defective adenoviral vectors having at least a non-functional E1 region and comprising a Ki gene, under the control of a transcriptional promoter,
- replication-defective adenoviral vectors having at least a non-functional E1 and E4 regions and comprising a Ki gene, under the control of a transcriptional promoter,
- replication-defective adenoviral vectors lacking all endogenous viral genes (gutless or minimal vectors) and comprising a Ki gene, under the control of a transcriptional promoter,
- replication-defective AAV vectors lacking functional Rep and Cap regions and comprising a Ki gene, under the control of a transcriptional promoter,

Other preferred vectors are plasmids, such as pCOR plasmids comprising a conditional origin of replication (WO97/10343) or any other plasmid preferably lacking an antibiotic-resistance gene or a fully functional origin of replication. Such plasmids may be obtained or

prepared according to conventional methods.

The Ki gene may be placed under the control of any suitable transcriptional promoter, preferably a promoter functional in mammalian cells, more preferably in human cells. The promoter may be constitutive, ubiquitous, regulated, inducible, tissue-specific, etc. The choice of a particular promoter can be made by the skilled person, depending on the cell to be treated.

Specific examples of promoters suitable for use in the present invention include promotersof viral origin (such as cytomegalovirus, respiratory syncitial virus, rous sarcoma virus, simain virus 40, retrovirus or lentivirus and the likes known in the states of art) of mammalian origin , house-keeping promoters (such as EF1alpha or E2F) or specifically expressed among which promoters of VEGF(s), FGF(s), HIF(s), Tie, VE cadherin, creatin kinase M, desmin, PGK, alpha smooth muscle cell actin, SM22, steel , kit, cardiac myosin, alpha myosin heavy chain, neuronal specific promoters and all promoter from all similar genes known by the skilled in the art person. Promoters could also be of synthetic origin such as inducible or regulated promoters (modulated by tetracycline, rapamycin, PPAR activator, hormone analogs, physiopathological situations...) or any promoters derived from fragments of the above mentioned using techniques known by the skilled person (deletion mutants, chimeric promoter, ...).

These vectors, particularly the above viral vectors, represent specific objects of the preent invention. Also, any cell containing a vector as denied above is the object of this invention, particularly cells infected with a viral vector as defined above. The invention also encompasses methods of producing viral vectors as defined above, comprising introducing a viral vector comprising a Ki gene in a packaging cell, and isolating the viruses prepared.

The present invention may be used to treat essentially any cell that is subject to natural or induced or provoked apoptosis. This includes various cell types, either isolated or in tissues, cultures, suspensions, organs or whole organisms, in healthy or pathological condition, such as myocardial cells (preferentially cardiac, skeletal or smooth muscle cells), muscle

cells, fibroblasts, hepatic cells, endothelial cells, nervous cells, etc. The invention is particularly suited to treat (cardiac) muscle cells or nervous cells, in particular damaged or altered (cardiac) muscle cells or nervous cells.

In this regard, the present invention can be used for the treatment or prevention of disorders resulting from excessive cell death that affect muscle cells, or cardiovascular vessels including but not limited to myocardial, cerebral or peripheral ischemia, infarct, heart failure, aneurisms and also non cardiovascular disorders such as inflammatory diseases, neurodegenrative diseases, stroke, ulcers, infertility, scleroderma, renal diseases, eye diseases, hepatic diseases and pulmonary disesases . This invention also finds utility for the treatment of general disorders due to excessive cell proliferation such as atheroma, retinopathy, cancers, metastasis, AIDS complications, abnormal skin proliferation, psoriasis or rhumatoid arthritis.

Particular objects of this invention reside in methods of treating a subject with heart disease, comprising administering to the subject, preferably through direct myocard or heart injection, an amount of a Ki gene or polypeptide, preferably a Ki gene, efficient to ensure protective activity of myocardial cells exposed to said gene or polypeptide.

The invention is also suitable to treat various nervous cells, to protect said cells from death or degeneration. According to this embodiment, this invention can be used to prevent or treat or reduce the burden of subjects with neurodegenerative disorders (ALS, SMA, Parkinson's disease, Alzheimer's disease, etc.).

In this regard, the invention relates to a method of preventing or treating or reducing the burden of a subjects with neurodegenerative disorder, comprising administrering to the subject an amount of a Ki gene or polypeptide, preferably a Ki gene, efficient to ensure protective activity of nervous cells exposed to said gene or polypeptide.

In these embodiments, the Ki gene or polypeptide may be used either alone or in combination with additional agents or treatments, in particular agents or treatments used for cardiovascular or neurological indications.

These include for instance, for cardiovascular indications, anticoagulant factors, such as tPA, heparin, low molecular weight heparin, growth factors including angiogenic factors or insulin like growth factors. In general, the Ki gene or polypeptide may be associated with other treatments as known in the state of the art for the indication, or known to enhance the desired effect of the Ki-mediated treatment by the skilled person.

In this respect, a particular object of this invention resides in a composition comprising a Ki gene or polypeptide and a second therapeutic or prophylactic agent, for simultaneous, separate or sequential use. The composition may comprise any pharmaceutically acceptable carrier or vehicle or excipient, such as saline buffer, isotonic solution, additives, stabilizers, etc. The second agent may be a cyto-protective agent, an (anti-)coagulation factor, an (anti-)platelet agent, etc.

The above polypeptides, genes or compositions may be used in different ways. For in vitro or ex vivo uses, they may be contacted directly with the cells, in any appropriate device (tube, plate, dish, pouch, etc.). For in vivo uses, they may be administered to a subject in need thereof, by various conventional routes such as local injection, intracardiac injection, pericardial injection, intra-tumoral injection, stereotaxic injection, intra-muscular injection, percutaneous injection, etc. It is believed that the specific administration protocol, including route, dose and frequency, may be adjusted by the clinician, depending on the situation. Typically, for administration of a virus, 10^4-10^{13} viral particles may be injected per dose, more generally between 10^6-10^{12}.

Another object of this invention resides in a method of causing or stimulating (or enhancing) apoptosis in a cell, more particularly in a tumor cell or a smooth muscle cell,

comprising contacting the cell with a compound that inhibits Ki gene expression or activity. Based on the presently demonstrated protective role of Ki, the invention also provides novel strategies to cause cytotoxicity or apoptosis, or to enhance cytotoxicity or apoptosis in a cell.

The compound can be, in particular embodiments, an antisense nucleic acid molecule that inhibits Ki gene expression, an anti-Ki antibody, or a chemical inhibitor or competitor, including peptides (or nucleic acids encoding the same).

In a particular embodiment, the compound is an antisense nucleic acid molecule specific for Ki gene (as defined above, including any transcription product thereof). These antisense molecules can be targeted against the genomic DNA with which they can form a triple helice, or against any transcription product of said genomic DNA, including messenger RNAs, with which they form double helices. Preferred antisense molecules comprise all or part of SEQ ID NO:1 (or its complementary sequence) and are directed against Ki mRNA. A particular type of antisense molecules comprises synthetic single-strand oligonucleotides comprising between 5 and about 100 bases, more preferably between 5 and 40 bases and having a sequence region that is complementary to at least a region of a Ki RNA.

The antisense molecules may be produced by any conventional technique, including artificial nucleic acid synthesis and/or recombinant DNA techniques (isolation from DNA libraries, cleavage, ligation etc.). The antisense molecules may contain non-natural bases and/or may be chemically modified to increase their stability (or the stability of the hybrid) and/or to induce a degradation of the hybrid formed. In this regard, the antisense molecule may also be a Peptide Nucleic Acid molecule (PNA), i.e., an antisense nucleic acid comprising one or several amino acids. The antisense molecules of this invention may also comprise double stranded DNAs encoding antisense oligonucleotides as described above, as well as ribozymes.

In a particular embodiment, the present invention thus resides in a method of causing or

stimulating apoptosis in a cell, comprising contacting said cell with an antisense nucleic acid molecule specific for Ki gene or any transcription product derived therefrom.

The compounds that inhibit Ki gene expression or activity can also be a peptide, preferably a peptides of 5 to 50 amino acid resides, even more preferably 5 to 25, or a nucleic acid encoding the same. The peptide preferably comprises a portion of Ki polypeptide and is able to interfere with the activity of Ki, in particular its binding to Gax and/or p53.

Other compounds that may be used to inhibit Ki expression or activity are antibodies, as well as fragments and derivatives thereof. Antibodies may be polyclonal, monoclonal, recombinant, , etc. Antibody derivatives or fragments include any portion of an antibody (e.g., Fab, Fab'2, CDR, etc.) that retains at least a potion of the epitope or antigen-specificity thereof. Derivatives also include ScFv, which can be synthesized directly into a target cell.

More generally, the compounds that inhibit Ki gene expression or activity include any natural or artificial product that is able to inhibit Ki gene expression or Ki polypeptide activity. These include any product interfering with the interaction between a Ki polypeptide and a Gax or p53 protein. Such product may be a peptide, nucleic acid, lipid, sugar, inorganic or organic substance, whether natural, synthetic or semi-synthetic.

Furthermore, in a specific embodiment, the compound is used either alone or in combination(s) with additional cytotoxic agents, such as tumor suppressor genes or polypeptides, anti-tumor chemical agents or treatments, etc. Specific examples of tumor suppressor genes include any nucleic acid or polypeptide selected from the p53 tumor suppressor product, the Rb tumor suppressor product, etc. Cytotoxic agents include any anti-tumor agent such as, for instance, Taxol, taxotere, DNA damaging agents (cis-platinum, etc), cytotoxic gene therapy and the like. The cytotoxic treatment also includes radiotherapy. The cytotoxic agent or treatment may be used following conventional

protocols, before, after or together with the compound that inhibits Ki gene expression or activity.

In this regard, a further aspect of this invention resides in compositions comprising a compound that inhibits Ki gene expression or activity and a cytotoxic agent, for simultaneous, separate or sequential use. As mentioned above, the composition may contain any pharmaceutically acceptable carrier or vehicle or excipient, such as saline buffer, isotonic solution, additives, stabilizers, etc.

This embodiment of the present invention may be used to regulate apoptosis in various cell types as mentioned above, and is particularly advantageous to cause or stimulate apoptosis in a tumor cell or a smooth muscle cell.

The above polypeptides, genes or compositions may be used in different ways. For in vitro or ex vivo uses, they may be contacted directly with the cells, in any appropriate device (tube, plate, dish, pouch, etc.). For in vivo uses, they may be administered to a subject in need thereof, by various conventional routes such as local injection, systemic injection, intra-tumoral injection, intracardiac or pericardial (stereotaxic) injection, intra-muscular injection, percutaneous catheter-mediated delivery (in particular for arterial smooth muscle cells to block restenosis), etc. It is believed that the specific administration protocol, including route, dose and frequency, may be adjusted by the clinician, depending on the situation. Typically, for administration of a virus, 10^4-10^{13} viral particles may be injected per dose, generally between 10^6-10^{12}.

Additional aspects of this invention include compositions, such as pharmaceutical compositions, nucleic acid constructs, recombinant cells as well as methods of treating or preventing various pathological conditions where abnormal cell cycle regulation occurs, such as tumors, neuro-degeneration, muscular hyperproliferative disorders, fibrosis, arterial, stenosis, myocardial disorders, hepatitis, inflammatory diseases, etc.

Further aspects and advantages of the present invention will be described in the following experimental section, which should be regarded as illustrative and not limiting the scope of the present application. All citations are incorporated to this application by reference.

Materials And Methods

Cell culture

COS-1 cells were obtained from ATCC (American Type Culture Collection) and were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). COS-1 cell is a fibroblast-like cell line established from CV-1 simian cells which were transformed by an origin-defective mutant of SV40 which codes for wild-type antigen.

Tumoral H1299 and H460 cells derived from non small cell lung cancer, were obtained from ATCC (American Type Culture Collection) and were grown in RPMI 1640 medium (Gibco BRL, Cergy Pontoise, France) supplemented with 10% fetal bovine serum and penicillin (100mM), streptomycin (100 µg/ ml), L-glutamin (2 mM) (Gibco BRL, Cergy Pontoise, France). Cells were grown at 37°C with 95% humidity and 5% CO2.

Plasmid and recombinant protein production

The plasmid pCM 282 coming from the lung cDNA library (Clontech, Palo Alto, USA) and including gal4TA and Ki in fusion, was digested by Nco I et Xho I to obtain Ki. Then Ki was ligated with the plasmid pAS1 (McArdle Laboratory for cancer Research, University of Wisconsin-Madison, Madison, WI 53706) digested by Nco I/Sal I. The plasmid TagHAKi obtained was digested by EcoR I/dra I and ligated in pCDNA3 in EcoR I/EcoR V.

The GST-Gax protein production was realized from plasmid pGEX-2T-hGax. Plasmids were transformed into BL21 (Amersham Pharmacia Biotech, England) for expression. The cells were grown at 37°C in LB medium with ampicillin. The cultures were grown to an A600 between 0.6 and 0.8 and then induced with 0.1 mM isoprppyl-b-thiogalactoside

(IPTG). Cells were sonicated and the purification of GST-GAX protein was realized on agarose-beads coupled to glutathion.

Ki-myc cloning was realized in pET-29 plasmid (Novagen) from PGAD (Clontech, Palo Alto, USA) contained in the yeast. PET-29 plasmid produces the protein fused to the S-tag epitope). Plasmids were transformed into BL2, the cultures were grown to an A600 between 0.6 and 0.8 and then induced with 0.1 mM isopropyl-b-thiogalactoside (IPTG) and T7 RNA polymerase induced by BL-21. Cells were sonicated and the purification of SmycKi protein was realized on agarose-beads coupled to S-protein.

Transfection

Cos-1 cell transfections were performed in a 4 wells culture dish at a density of 10 000 cells in in DMEM supplemented with 10% fetal calf serum (FCS) and transfected.

H1299 cell transfections were performed in 24 well (Falcon) at a density of 100 000 cells in DMEM supplemented with 10% fetal bovine serum by using lipofectamine (Gibco BRL Cergy Pontoise, France) at a ratio 4 / 1 (lipofectamine/ADN). After a 4 h incubation in serum free medium, medium was changed to complete medium which contained 10% serum and cells were maintained in culture for 24 h to 72 h depending on the assay.

Crystal violet method

72 hours post infection, cell viability was measured using crystal violet: cells are fixed by formaldehyde 4% (diluted in H2O) for 30 minutes at room temperature, Cristal Violet at .0.1% was then added for 20 minutes at room temperature. Two washes with H2O were necessary to remove extracellular Cristal Violet. Cells made soluble in sodium deoxycholate 2% preheated at 37°C. An incubation for 30 minutes was done under agitation before transferring 100 µL of soluble fraction in a 96 wells plate in order to measure D.O at 550 nm.

Results

Ki counteracts gax and p53 mediated tumor cell death (Figure 1)

This example demonstrates the effects of Ki antigen on cell viability, in the presence or absence of Gax. We performed a transfection-based assay in the H1299 cell line that examines the relative frequency of surviving cells by using the crystal violet method 72 h post transfection. We observed that Ki alone does not exhibit any toxic activity or growth-stimulatory activity in our experimental conditions, like the control plasmid. On the contrary, transfection of a plasmid encoding Gax decreased cell viability by 35% to 40%. Surprisingly, when gax and ki were simultaneously expressed, we did not observe cell death. Ki is thus able to counteract gax mediated cell death. The p53 tumor suppressor gene induces apoptosis in a number of different cell types (Elledge and Lee 1995; White 1996). We determined whether Ki could affect p53-induced cell death. Using the same conditions of transfection, we observed that the transfection of p53 encoding plasmid leads to up to 50% decrease in cell viability. Surprisingly, p53 induced cell death was not observed in the presence of ki. These data thus show that Ki is able to counteract gax and p53 induced cell death.

We then tested whether gax and Ki interaction interferes with gax growth arrest in the tumoral cell line H1299. We showed that Gax cytotoxicity is inhibited in the presence of Ki antigen. Furthermore, while transfection of p53 encoding plasmid led to around 50% decrease of cell viability, as expected, p53 induced cell death was surprisingly not observed in the presence of Ki antigen. Therefore, these data provide evidence that Gax or p53 association with Ki is a key feature of the apoptotic process and that increasing Ki activity is a novel and efficient method to block apoptotic pathways.

Ki counteracts gax-mediated fibroblast cell death (Figure 2)

Triplicate cultures of p53-/- murine fibroblasts (MEFs) were cultured on 60 mm plates containing 1.5% gelatin coated glass coverslips and were serum-starved for three days in 0.5% FBS/DMEM. 4 μg of test plasmid (Gax or Ki) were co-transfected with 1 μg of CMV-b-Gal expression plasmid using the Lipofectamine procedure (GobcoBrl) in a 1:6

ratio, for 2-4 hours. Cultures were serum stimulated for 48 hours and fixed in 4% neutral buffered formalin for 10 minutes. Plasmids pCDNA-bcl-2, pCDNA-bcl-x and pCDNA-bax were previously described. Plasmid pCMV-HA-KI comprises the HA-tagged Ki gene under the control of the CMV promoter. Values represent the mean and standard eror, and were compared for statistical significance by Anova analysis and student t-test ($p<0.05$).

The results, presented Figure 2, demonstrate that (i) Ki gene or polypeptide does not kill the cells while Gax or bax induce a very significant cell death and (ii) co-expression of Ki with Gax can reverse Gax-induced cell death.

These results thus confirm the cyto-protective activity of Ki in fibroblast cells, and further support the novel and dominant role of Ki as a cytoprotective factor.

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CLAIMS

1. A method of inhibiting or reducing apoptosis in a cell, comprising contacting the cell with a Ki polypeptide or gene.

2. The method of claim 1, for inhibiting or reducing p53-mediated apoptosis.

3. The method of claim 1, for inhibiting or reducing GAX-mediated apoptosis.

4. The method of claim 1, wherein the cell is a cardiomyocyte.

5. The method of claim 1, wherein the cell is a muscle cell.

6. The method of claim 1, wherein the cell is a neuronal cell.

7. The method of claim 1, comprising contacting the cell, in vivo or ex vivo, with a vector encoding a Ki polypeptide.

8. The method of claim 7, wherein the vector is a viral vector.

9. The method of claim 7, wherein the vector is a plasmid.

10. A method of treating a cell to protect said cell from death or degeneration, comprising contacting the cell with a Ki polypeptide or gene.

11. A method of causing or stimulating apoptosis a cell, comprising contacting the cell with a compound that inhibits Ki gene expression or activity.

12. The method of claim 11, wherein the compound is an antisense nucleic acid molecule

that inhibits Ki gene expression.

13. The method of claim 11, wherein the cell is further exposed to a cytotoxic agent, before, after or together with the compound that inhibits Ki gene expression or activity.

14. The method of claim 13, wherein the cytotoxic agent is a tumor suppressor gene or polypeptide.

15. The method of claim 13, wherein the cytotoxic agent is a anti-tumor chemical agent.

16. A viral vector comprising a Ki gene.

17. The vector of claim 16, which is an adenoviral vector.

18 The viral vector of claim 16, which is an AAV-derived vector.

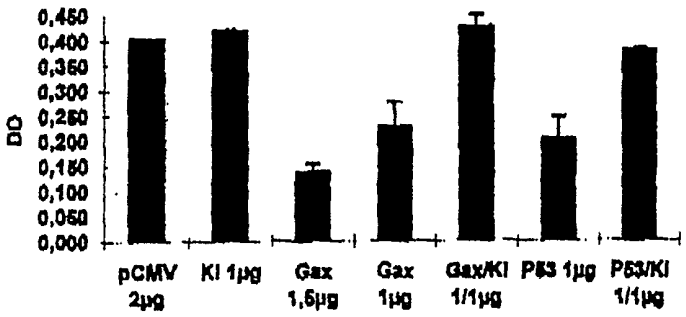
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Figure 1

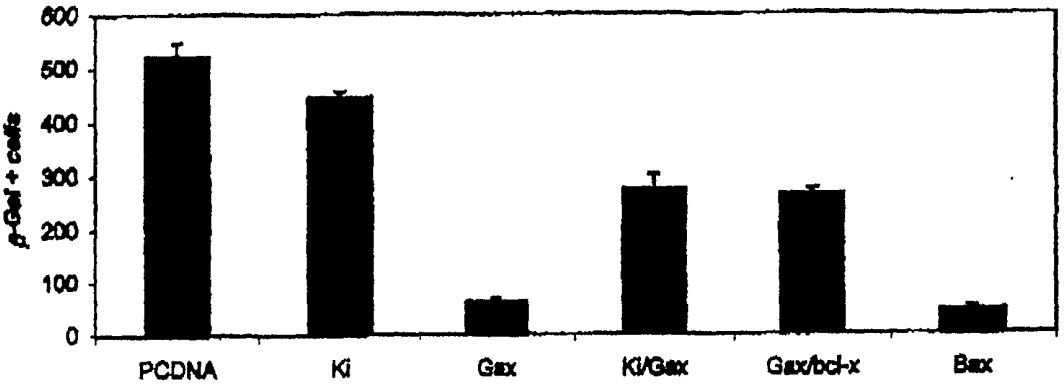
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Figure 2

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