Rapamycin reverses NPM-ALK-induced glucocorticoid resistance in lymphoid tumor cells by inhibiting mTOR signaling pathway, enhancing G₁ cell cycle arrest and apoptosis

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The anaplastic lymphoma kinase (ALK) is an oncogene product involved in hematopoietic and non-hematopoietic malignancies. Recent studies have demonstrated that nucleophosmin (NPM)-ALK, originated from the fusion of NPM and ALK genes, causes cell transformation through diverse mechanisms. Here, we show a novel mechanism by which NPM-ALK transforms lymphoid tumor cells to become resistant to glucocorticoid (GC) or dexamethasone (DEX) treatment. Transformed BaF3 cells by NPM-ALK were much more resistant to DEX compared with their parental cells, and concurrently had a constitutive activation of mammalian target of rapamycin (mTOR) signaling, as evidenced by hyperphosphorylation of its downstream effectors, p70 S6 kinase (p70S6K) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1). The mTOR inhibitor rapamycin suppressed activation of p70S6K in BaF3/NPM-ALK cells and reversed GC resistance by synergistically inhibiting mTOR signaling pathway, enhancing G₁ cell cycle arrest and apoptosis.

Introduction

Glucocorticoids (GCs), such as prednisolone and dexamethasone (DEX), can induce cell cycle arrest and apoptosis in lymphoid cells, and therefore for decades constitute the first choice almost among all of the chemotherapeutic regimens for the treatment of lymphoid malignancies that include non-Hodgkin’s lymphoma, acute lymphoblastic leukemia (ALL), multiple myeloma and chronic lymphocytic leukemia, but most successfully for the treatment of newly diagnosed childhood ALL.² However, GC resistance occurs in 10–30% of untreated ALL patients, much more frequently in adult ALL and in relapsed ALL,³⁻⁵ and it is clinically the main cause of treatment failure in ALL.⁶ Thus, defining the molecular mechanism and finding a way to overcome it would contribute to the improvement of the outcome of the patients.⁷⁻⁸

Anaplastic lymphoma kinase (ALK) was first discovered by Dr Steve Morris’s group in 1994 because of its involvement in the (t(2;5)) chromosomal translocation that occurs in about two-thirds of anaplastic large cell lymphoma (ALCL), a rare type of non-Hodgkin’s lymphoma.⁹ The normal ALK gene encodes a receptor tyrosine kinase of insulin receptor superfamily, which contains a number of tyrosine kinases known to be involved in oncogenesis.¹⁰ The normal functions of ALK are not yet completely clear, despite robust expression of ALK in the developing central and peripheral nervous systems.¹¹ Dysregulation of ALK (almost exclusively caused by fusion to its partners) is a causative factor in ALCL and a subset of diffuse large B-cell non-Hodgkin’s lymphoma, and in non-hematopoietic neoplasms, including inflammatory myofibroblastic tumor, esophageal cancer and non-small cell lung cancer.¹²⁻¹⁵ ALK involvement in non-Hodgkin’s lymphoma occurs in the form of the NPM-ALK chimeric protein formed by the t(2;5) in ~75% of ALK-positive lymphomas; the other 25% express variant fusions that, such as NPM-ALK, contain the entire cytoplasmic portion of ALK, but fuse to other N-terminal partners. To date, at least 15 ALK fusions, namely, AL017-ALK, ATIC-ALK, CARS-ALK, CLTC-ALK, EML4-ALK, MSN-ALK, MYH9-ALK, NPM-ALK, RANBP2-ALK, SEC31L1-ALK, TFG-ALK(L), TFG-ALK(S), TFG-ALK(XL), TMP3-ALK and TMP4-ALK, have been identified,¹⁶ but no NPM-ALK has been so far found in non-hematopoietic malignancies. The underlying mechanism for abnormal activation of ALK fusions is that the N-terminal partner encodes an oligomerization motif that functions to activate the kinase domain of the ALK segment, which is critical for oncogenesis. NPM-ALK and variant ALK fusions are potent oncoproteins capable of transforming a wide variety of cell types in vitro, including rodent fibroblasts, such as NIH-3T3, Fr3T3 and Rat-1, and hematopoietic cells such as the myeloid line 32Dcl3 and murine pro-B lymphoid line BaF3.¹⁷ Interestingly, we found that transformed BaF3 cells not only could grow in the absence of interleukin (IL)-3, but also gained resistance to Dex-induced cell death.

The mechanisms of cell transformation mediated by the NPM-ALK tyrosine kinase are not fully understood. However, evidence suggests that activation of mammalian target of rapamycin (mTOR) signaling pathway contributes to tumor cell survival in ALK+ ALCL cell lines and tumors.¹⁸⁻¹⁹ mTOR is a serine/threonine protein kinase that has an important function in regulating protein synthesis, cell cycle progression and cell proliferation.²⁰ The activation of PI3K/Akt/mTOR pathway is common in many cancers, and can occur through multiple mechanisms, including mutation or decreased expression of the tumor suppressor PTEN, mutation or amplification of PI3K, amplification of Akt and activation of receptors or oncogenes upstream of the PI3K/Akt/mTOR pathway.²¹ A recent study, using a database of drug-associated gene expression profiles to...
screen for molecules whose profile overlapped with a gene expression signature of GC sensitivity/resistance in ALL cells, has demonstrated that the mTOR inhibitor rapamycin profile matched the signature of GC sensitivity. This led us to hypothesize that NPM-ALK induces GC resistance in lymphoid cells through activation of mTOR signaling pathway.

We tested the phosphorylation status of mTOR (p-mTOR) in 44 ALK+ ALCL tumors and 6 t(2;5) ALCL cell lines, and found that p-mTOR was detected in 39 (89%) cases of ALK+ ALCL tumors and all ALK+ ALCL cell lines, suggesting that NPM-ALK may activate mTOR signaling pathway. In this study, we demonstrate that NPM-ALK can induce mTOR activation in transformed BaF3 cells, and rapamycin can reverse NPM-ALK-induced GC resistance in lymphoid cells by synergistically inhibiting mTOR activity, enhancing G1 cell cycle arrest and apoptotic cell death, but not by modulating the expression of GC receptor (GR).

Materials and methods

Cell lines

ALK+ ALCL cell line Karpas299, murine pro-B lymphocyte BaF3 and NPM-ALK transformed BaF3 cells were kindly provided by Dr Steve Morris (St Jude Children’s Research Hospital, Memphis, TN, USA). BaF3/NPM-ALK and Karpas299 were maintained in RPMI 1640 (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Sigma, St Louis, MO, USA), 2 mm L-glutamine (Gibco) and antibiotics (penicillin 100 U/ml and streptomycin 50 µg/ml) at 37 °C in a humidified 5% CO2 in-air atmosphere. BaF3 cells were cultured in the same media but with 10 ng/ml IL-3.

Reagents and antibodies

Rapamycin (Calbiochem, La Jolla, CA, USA) was dissolved in dimethyl sulfoxide (Sigma) and used at the concentration of 10 nm. Dex (Sigma) was dissolved in ethanol and used at the concentration of 1 µM. The final concentrations of dimethyl sulfoxide and ethanol in the medium were 0.05 and 0.1%, respectively, at which cell proliferation/growth or viability was not obviously altered (data not shown). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma, St Louis, MO, USA), Bim, Mcl-1, Cyclin A, caspase-3, NF-κB, cyclin D1, p21kip1, Bax, Bcl-2 (Cell Signaling Technology, Beverly, MA, USA), p21waf1 (BD Bioscience, San Jose, CA, USA), Bim, Mcl-1, Cyclin A, caspase-3, NF-kB (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and GAPDH (KangChen Bio-Tech, Shanghai, China) were used. Horseradish peroxidase-conjugated donkey anti-rabbit IgG and horseradish peroxidase-conjugated sheep anti-mouse IgG were obtained from Santa Cruz Biotechnology.

Cell treatment

Logarithmically growing cells were harvested and replaced in 96- or 6-well sterile plastic culture plates (Corning, Corning Inc., Acton, MA, USA) to which 10 nm rapamycin (Rap group), 1 µM Dex (Dex group), 10 nm rapamycin plus 1 µM Dex (Rap + Dex group), or 0.05% dimethyl sulfoxide plus 0.1% ethanol (control group) was added, respectively. At the end of the incubation, cells were transferred to sterile centrifuge tubes, pelleted by centrifugation at 400 g at room temperature for 5 min and prepared for analysis as described below.

Proliferation assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay is based on the conversion of the yellow tetrazolium salt to purple formazan crystals by metaboliclly active cells and provides a quantitative estimate of viable cells. Cells were seeded in 96-well plates (20 000 per well), incubated for 48 h. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (0.5 mg/ml, final concentration) was added to each well for 4 h at 37 C. Then, 100% (v/v) of a solubilization solution (10% SDS in 0.01 mol/l HCl) was added to each well, and the plates were re-incubated for 24 h at 37 °C. Spectrophotometric absorbance was measured at 570 nm (reference 690 nm) using a multiplate reader (Multiskan Spectrum, Thermo Electron Co., Vantaa, Finland). Values were obtained by comparing these cells with their respective controls.

Assay for apoptosis

The samples were washed with phosphate-buffered saline and resuspended in 500 µl of binding buffer containing 5 µl of AnnexinV-FITC stock and 5 µl of propidium iodide for determination of phosphatidylserine exposure on the outer plasma membrane. After incubation for 10 min at room temperature in a light-protected area, the samples were quantified by flow cytometry (FASCAria, BD Bioscience, San Jose, CA, USA).

Cell cycle analysis

Approximately, 106 cells were collected and fixed overnight in 70% ethanol at 4 °C. Cells were washed and stained with 5 µg/ml propidium iodide in the presence of DNase-free RNase (Sigma). After 30 min at room temperature, the cells were analyzed by flow cytometry (Beckman Coulter Inc., Miami, FL, USA).

Western blotting analysis

Cells (106) were washed twice in cold phosphate-buffered saline, and then lysed by Laemmli sample buffer (Bio-Rad, Hercules, CA, USA). Samples were boiled for 5 min at 100 °C. Proteins were separated on 10 or 15% SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (0.45 µm, Millipore, Sao Paulo, SP, Brazil). Nonspecific-binding sites were blocked with 5% nonfat dry milk dissolved in TBS (10 mM Tris-HCl, pH 7.6, 137 mM NaCl) with 0.1% Tween 20 (TTBS) for 1 h at room temperature followed by incubation with the primary antibodies at 4 °C overnight. The membranes were washed three times in TTBS and incubated for 1 h at room temperature with the corresponding secondary antibodies diluted in 5% nonfat dry milk. Proteins were visualized by ECL plus (Amersham Biosciences, Oxford, UK). All experiments were carried out independently at least three times. The level of the GAPDH protein was used as a control of the amount of protein loaded into each lane.

Statistical analysis

All assays were performed in triplicate, and data are shown as mean ± s.d. Statistical significance of differences between groups was determined using the Student’s t-test. Results were considered significant with P-values <0.05.

Results

Expression of NPM-ALK confers resistance to GC treatment in lymphocytes

NPM-ALK confers resistance to GC treatment in lymphocytes. Nonmalignant murine pro-B cells (BaF3) were stably transfected with the cDNA encoding NPM-ALK. Expression of NPM-ALK...
converted BaF3 cells from IL-3 dependent to the cytokine independent in growth. Surprisingly, expression of NPM-ALK also conferred resistance to Dex treatment in the cells (Figure 1a). Even under higher concentrations (>500 μM), BaF3/NPM-ALK and NPM-ALK human ALCL cell line Karpas299 could still grow and proliferate compared with empty vector transfected BaF3 cells.

**Enforced expression of NPM-ALK upregulates mTOR pathway in transfected BaF3 cells**

We previously tested mTOR status in six ALK+ ALCL lines and found that mTOR pathway was activated in all of them. Here, we used Karpas299 as a positive control to check the activation status of mTOR pathway in transformed BaF3/NPM-ALK cells. Our data showed that highly phosphorylated 4E-BP1 and p70S6K were detected (Figure 2), suggesting that mTOR pathway was activated. BaF3 cells growing with IL-3 also exhibited activation of mTOR, since IL-3 itself can activate PI3K/ AKT/mTOR pathway. At 1 h after withdrawal of IL-3, there was almost no detectable phospho-4E-BP1 and phospho-p70S6K in parental BaF3 cells (Figure 2), indicating that activation of mTOR pathway was mainly induced by NPM-ALK in the IL-3-independent BaF3/NPM-ALK cells.

**Rapamycin and Dex synergistically inhibit growth of ALK+ lymphoid cells**

When the ALK+ lymphoid cells, BaF3/NPM-ALK and Karpas299, were treated with rapamycin or Dex alone, each had some inhibition on the growth of the cells. However, when in combination, rapamycin and Dex synergistically inhibited the growth of all cells, especially in BaF3/NPM-ALK. A strong synergistic inhibition of mTOR signaling demonstrated by dephosphorylation of phospho-p70S6K by rapamycin and Dex. To evaluate the effect of rapamycin and Dex on mTOR signaling, Western blot analysis was performed. We found that rapamycin or Dex, to some extent, inhibited phosphorylation of 4E-BP1 and p70S6K in the cells. However, when they were used together, the mTOR activation was almost completely blocked in all cells (Figure 3b), suggesting a strong synergistic inhibitory effect of rapamycin and Dex on mTOR signaling.
Inhibition of mTOR induces G1 cell cycle arrest in ALK+ lymphoid cells

Rapamycin or Dex induced G1 cell cycle arrest in the lymphoid cells tested. When the two drugs were used simultaneously, more cells were arrested in G1 phase (Figure 4a). To evaluate the molecular basis underlying cell cycle arrest, cell cycle regulatory proteins were probed. Following exposure to rapamycin or Dex alone for 48h, the cyclin-dependent kinase inhibitors p21waf1 and p27kip1 were found to be upregulated in all cells, and there were some synergistic induction of p21waf1 and p27kip1 in the cells when rapamycin and Dex were in combination. Rapamycin did not obviously affect the expression of Cyclin A, whereas Dex induced Cyclin A expression to some extent. Rapamycin prevented Dex-induced expression of Cyclin A; Cyclin D1 levels were reduced when treated with rapamycin or Dex alone or in combination. In particular, when the cells were treated with the two drugs simultaneously, Cyclin D1 levels dropped very sharply (Figure 4b).

Inhibition of mTOR pathway sensitizes ALK+ lymphoid tumor cells to apoptosis induced by Dex

We studied the apoptotic rate in the cells after 48h exposure to rapamycin or Dex alone, or in combination by flow cytometry. Rapamycin or Dex alone induced considerable levels of apoptosis in BaF3 and Karpas299 cells, but not in BaF3/NPM-ALK cells. However, when in combination, rapamycin and Dex induced significant apoptosis in all cells. The apoptotic rate increased by 4.2-fold in BaF3 cells, 4.1-fold in BaF3/NPM-ALK and 2.5-fold in Karpas299 cells, respectively (Figure 5a).

To understand the mechanism by which rapamycin and Dex induced apoptosis of the cells, we investigated the effects of rapamycin and Dex on the expression of apoptosis-related proteins. Bcl-2 level remained unchanged after 24h exposure to rapamycin or Dex alone or in combination, and a little upregulated after 48h treatment with rapamycin and Dex together in all three cells, especially in GC-resistant ALK+ BaF3/NPM-ALK and Karpas299 cells. However, Mcl-1 expression was inhibited by rapamycin in all three cells, and most strikingly, there was a marked synergistic inhibition of Mcl-1 when rapamycin and Dex were used together, especially in NPM-ALK transformed BaF3 cells. The proapoptotic protein Bax was induced by rapamycin alone or in combination with Dex after 48h treatment. Dex alone had very little induction of Bax. Bim was induced by rapamycin or Dex alone or in combination in GC-sensitive parental BaF3 cells, but was only induced by combination of rapamycin and Dex in GC-resistant BaF3/NPM-ALK and Karpas299 cells (Figure 5b).

These results suggest that rapamycin sensitized Dex-induced apoptosis in ALK+ GC-resistant lymphoid tumor cells by enhancing proapoptotic gene expression of Bax and Bim, and inhibiting antiapoptotic gene expression of Mcl-1 but not Bcl-2.
various fields. The inhibitors of mTOR have been extensively studied in clinical trials for treatment of different types of cancer. With developing novel inhibitors of ALK kinase, addition of mTOR inhibitors to the current treatment protocol for ALK+ tumors may predict an even better long time survival and cure rate.

Discussion

It is very well established that NPM-ALK and variant ALK fusions transform lymphocytes and other cells by constitutively activating cell-signaling pathways leading to cancer. Increasing evidence suggests that rapamycin-sensitive mTOR pathway activated by NPM-ALK either through the mitogen-induced extracellular kinase/extracellular signal-regulated kinase signaling pathway or to a much lesser degree, through PI3K/Akt pathway, promotes malignant transformation. Here, we elucidated a crucial new function of fusion ALK tyrosine kinases that are expressed in both hematopoietic lymphomas and non-hematopoetic solid tumors. NPM-ALK induced transformed lymphocytes resistant to GC by activation of mTOR pathway. This is supported by the findings that constitutive hyperphosphorylation of p70S6K and 4E-BP1 was detected in BaF3/NPM-ALK cells and treatment of the cells with rapamycin inhibited mTOR signaling, and resensitized the cells to Dex.

Mammalian target of rapamycin pathway is constitutively activated in many types of cancers, including lymphoid malignancies, and is centrally involved in the control of cancer cell metabolism, growth, proliferation, angiogenesis, metastasis and drug resistance, which has attracted broad interest in various fields. The inhibitors of mTOR have been extensively studied in clinical trials for treatment of different types of cancer. So far promising results have been achieved in certain lymphomas that exclusively use GC in the chemotherapeutic regimens. The findings from this study and others indicate an important role of mTOR in induction of GC resistance and also reveal high potential of mTOR inhibitors in chemotherapeutics of lymphoid tumors.

Glucocorticoids specifically induce apoptosis in malignant lymphoblasts and are thus pivotal in the treatment of lymphoid malignancies, especially ALL. However, GC resistance is a therapeutic problem accounting for most of the treatment failures. The exact molecular mechanism remains poorly understood. Studies in the in vitro models suggest regulatory and/or structural defect in GR gene expression as a major cause for GC resistance in leukemia cells, but it is not always the case in patients. The fact that mTOR inhibitor rapamycin reverses GC resistance in lymphoid cells and activation mTOR pathway by expressing either a constitutively active NPM-ALK or Akt kinase induces GC resistance suggests an unquestionable role of mTOR signaling in GC resistance. Our studies demonstrate that rapamycin reverses NPM-ALK-induced GC resistance is not through modulation of GR expression, but through synergistic inhibition of mTOR signaling pathway and thus augmentation of G1 cell cycle arrest by synergistically inducing cyclin-dependent kinase inhibitors p21\textsuperscript{WAF1} and p27\textsuperscript{KIP1} and apoptotic cell death by upregulation of proapoptotic and downregulation of antiapoptotic gene expression.

Several antiapoptotic Bcl-2 family proteins have been shown to confer GC resistance in ALL cell lines and thymocytes from transgenic mice when overexpressed. However, only Mcl-1, but not Bcl-2 or Bcl-XL, is overexpressed in GC-resistant ALL cells from primary patients. In GC-resistant ALL cell line CEM-c1, rapamycin specifically reduced the expression of Mcl-1, but did not affect the levels of Bcl-2 and Bcl-XL. Reduction of Mcl-1 level by RNAi sensitized cells to Dex-induced apoptosis, whereas RNAi-mediated reduction of Bcl-2 had no effect on GC resistance. These data suggest that Mcl-1 is a key antiapoptotic protein governing GC resistance in ALL cells. In consistence with these findings, our studies demonstrate that rapamycin reverses NPM-ALK-induced GC resistance mainly through a mechanism of downregulation of antiapoptotic gene expression of Mcl-1 other than Bcl-2, and upregulation of proapoptotic gene expression of Bax and Bim.

Anaplastic lymphoma kinase fusion kinases are critical for the genesis of ALCL, although their pathogenic role in other tumors remains to be fully elucidated. Defining the mechanism of ALK fusion kinases-induced GC resistance through activation of mTOR pathway is, in particular, of therapeutic importance. With developing novel inhibitors of ALK kinase, addition of mTOR inhibitors to the current treatment protocol for ALK+ tumors may predict an even better long time survival and cure rate.

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References

Leukemia


