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Erythroid induction of K562 cells treated with mithramycin is associated with inhibition of raptor gene transcription and mammalian target of rapamycin complex 1 (mTORC1) functions

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ABSTRACT

Rapamycin, an inhibitor of mTOR activity, is a potent inducer of erythroid differentiation and fetal hemoglobin production in β-thalassemic patients. Mithramycin (MTH) was studied to see if this inducer of K562 differentiation also operates through inhibition of mTOR. We can conclude from the study that the mTOR pathway is among the major transcript classes affected by mithramycin-treatment in K562 cells and a sharp decrease of raptor protein production and p70S6 kinase is detectable in mithramycin treated K562 cells. The promoter sequence of the raptor gene contains several Sp1 binding sites which may explain its mechanism of action. We hypothesize that the G + C-selective DNA-binding drug mithramycin is able to interact with these sequences and to inhibit the binding of Sp1 to the raptor promoter due to the following results: (a) MTH strongly inhibits the interactions between Sp1 and Sp1-binding sites of the raptor promoter (studied by electrophoretic mobility shift assays, EMSA); (b) MTH strongly reduces the recruitment of Sp1 transcription factor to the raptor promoter in intact K562 cells (studied by chromatin immunoprecipitation experiments, ChIP); (c) Sp1 decoy oligonucleotides are able to specifically inhibit raptor mRNA accumulation in K562 cells. In conclusion, raptor gene expression is involved in mithramycin-mediated induction of erythroid differentiation of K562 cells and one of its mechanism of action is the inhibition of Sp1 binding to the raptor promoter.

Introduction

The mammalian target of rapamycin (mTOR) forms two complexes, named mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) which are regulated by phosphorylation, complex formation and localization within the cells [1–5]. mTORC1 is composed of mTOR, the regulatory associated protein of mTOR (raptor), mammalian LST8/G-protein β-subunit like protein (mLST8/GβL) and the recently identified partners PRAS40 and DEPTOR [6,7]. Raptor binds directly to mTOR signaling (TOS) motifs on downstream targets, including S6K1 (ribosomal S6 protein kinase 1) and 4EBP1 (eukaryotic initiation factor 4E-binding protein 1) as well as PRAS40 and Hif1α, thus linking them to the mTOR kinase [2,3]. mTORC1 senses and integrates diverse extra- and intracellular signals to promote anabolic and to inhibit catabolic cellular processes. This complex is characterized by the classic features of mTOR by functioning as a nutrient/energy/redox sensor and controlling protein synthesis [1,6]. The activity of this complex is stimulated by insulin, growth factors, serum, phosphatidic acid, amino acids (particularly leucine), and oxidative stress [6,8]. mTORC1 in yeast and mammals also promotes "ribosome biogenesis", a process whereby mTORC1 increases the transcription of ribosomal RNAs and proteins to augment cellular protein biosynthetic capacity [9–11]. mTOR Complex 2 (mTORC2) is composed of mTOR, rapamycin-insensitive companion of mTOR (Rictor), GβL, and mammalian stress-activated protein kinase interacting protein 1 (mSIN1) [2,3,12,13]. mTORC2 has been shown to function

Abbreviations: Raptor, regulatory associated protein of mTOR; Rictor, rapamycin-insensitive companion of mTOR; mTOR, mammalian target of rapamycin; mTORC1, mTOR complex 1; m-TORC2, mTOR complex 2; Sp1, specific protein 1; MTH, mithramycin; RAPA, rapamycin; ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; FBS, fetal bovine serum; PBS, phosphate-buffered saline; TBS, tris-buffered saline; HbF, fetal hemoglobin; ODN, oligonucleotide.

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as an important regulator of the cytoskeleton through its stimulation of F-actin stress fibers, paxillin, RhoA, Rac1, Cdc42, and protein kinase C α (PKCα) [14–17]. mTORC2 also appears to possess the activity of a previously elusive protein known as "PDK2." mTORC2 phosphorylates the serine/threonine protein kinase Akt/PKB at a serine residue S473. Phosphorylation of the serine stimulates Akt phosphorylation at a threonine T308 residue by PDK1 and leads to full Akt activation [13,16,17]; mTORC2 appears to be regulated by insulin, growth factors, serum, and nutrient levels [6,7]. Originally, mTORC2 was identified as a rapamycin-insensitive entity, as acute exposure to rapamycin did not affect mTORC2 activity or Akt phosphorylation. However, subsequent studies have shown that, at least in some cell lines, chronic exposure to rapamycin, while not affecting pre-existing mTORC2s, promotes rapamycin binding to free mTOR molecules, thus inhibiting the formation of new mTORC2 [14].

Despite the fact that the involvement of mTOR in all these and other biological processes has been firmly established, little information is available on the role of mTOR on erythroid differentiation. It has been demonstrated that rapamycin, an inhibitor of mTOR activity, is a potent inducer of erythroid differentiation of human leukemic K562 cells [18] and fetal hemoglobin production by β-thalassemic patients [19]. Accordingly, other inducers of K562 differentiation might operate through inhibition of mTOR [20–22]. In order to address this issue, mithramycin (MTH) was studied. This DNA-binding low molecular weight molecule is selective for G + C rich regions [23–25]. It binds to the minor groove of DNA generating unstable MTH-DNA complexes [20]. It is one of the most potent inducers of K562 differentiation [21] and HbF production by erythroid precursor cells from normal donors as well as β-thalassemia patients [26].

The main objective of the present study was to verify whether induction of differentiation by MTH is associated with inhibition of mTOR activity. The effects of MTH on raptor, rictor and mTOR gene expression were first analyzed by q-RT-PCR. Secondly, we determined by Western blotting, the production of mTOR, raptor and rictor proteins in MTH treated cells with the aim of determining whether mithramycin affects mTORC1, mTORC2 or both. Thirdly, we verified the possible effect of MTH on the interaction of the regulatory transcription factor Sp1 with the raptor promoter. This was approached by electrophoretic mobility shift assay, chromatin immunoprecipitation and treatment of target cells with Sp1 decoy molecules.

Materials and methods

Human K562 cell cultures

The human leukemia K562 [27,28] cells were cultured in a humidified atmosphere of 5% CO_2/air in RPMI 1640 medium (SIGMA, St. Louis, MO, USA) supplemented with 10% (vol/vol) fetal bovine serum (FBS; Biowest, Nuaille, F), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cell growth was studied by determining the cell number per ml with a Z2 Coulter Counter (Beckman Coulter, Fullerton, CA, USA).

Antibodies

The primary antibodies for Western blotting were: anti-Raptor cat. 2280, anti-Rictor cat. 2114, anti-mTOR cat. 2983, anti-p-mTOR (Ser2448) cat. 2971, anti-p-mTOR (Ser2481) cat.2974, anti-p70S6 kinase cat.2708, anti-p-p70S6 kinase (Thr389) cat.9234. All antibodies were purchase from Cell Signaling (Euroclone S.p.A., Pero, MI, Italy).

RNA extraction

Cells were isolated by centrifugation at 1500 rpm for 10 min at 4 °C, washed in PBS, lysed in Tri-reagentTM (Sigma–Aldrich, St. Louis, MO, USA), according to the manufacturer's instructions. The isolated RNA was washed once with cold 75% ethanol, dried and dissolved in diethylpyrocarbonate treated water before use.

Reverse transcription and quantitative real-time PCR (RT-qPCR)

The reagents for gene expression analysis by real-time RT-PCR were obtained from Applied Biosystems (Foster City, CA, USA). 500 ng of total RNA were reverse transcribed using random hexamers. RT-qPCR assay was carried out using gene-specific double fluorescently labeled probes. The primers and probes used to assay the expression of γ-globin, α-globin and mTOR mRNA were purchased from IDT (Integrated DNA technologies, San Jose, CA, USA). The nucleotide sequences used for real-time qPCR analysis of γ- and α-globin mRNAs and mTOR were as follows: α-globin forward primer, 5′-CAC GCG CAC AAG CTT CG-3′, α-globin reverse primer, 5′-AGG GTC ACC AGC AGG CAG T-3′, α-globin probe, 5′-FAM-TGG ACC CGG TCA ACT TCA AGC TCC T-TAMRA-3′; γ-globin forward primer, 5′-TGG CAA GAA GGT GCT GAC TTC-3′, γ-globin reverse primer, 5′-TCA CTC AGC TGG GCA AAG G-3′, γ-globin probe, 5′-FAM-TGG GAG ATG CCA TAA AGC ACC TGG-TAMRA-3′; mTOR forward primer 5′-GCT GTA CGT TCC TTC TCC TTC-3′, mTOR reverse primer 5′-CAA GAA CTC GCT GAT CCA AAT G-3′, mTOR probe, 5′-FAM-TGC ATT CCG-ZEN-ACC TTC TGC CTT CA-IABkFQ-3′. The nucleotide sequences used for real-time qPCR analysis of transferrin receptor and glycophorin A mRNAs were: transferrin receptor forward primer, 5′-TCA GAGCGTCGGGATATCG-3′, transferrin receptor reverse primer, 5′-TGA ACT GCC ACA CAG AAG AAC A-3′, transferrin receptor probe 5′-FAM-TGG CGG CTC GGG ACG GA-TAMRA-3′; glycophorin A forward primer, 5′-CGG TAT TCG CCG ACT GAT AAA-3′, glycophorin A reverse primer, 5′-AAA GGC AGT CTG TGT CAG GT-3′, glycophorin A probe, 5′-FAM-AAA GCC CAT CTG ATG TAA AAC CTC TTC CCC T-TAMRA-3′. The kit for quantitative RT-PCR for ζ-globin mRNA and ε-globin mRNA were from Applied Biosystems (ζ-globin mRNA: Hs00923579_m1; ε-globin mRNA: Hs00362216_m1). The primers and probes used to assay the expression of raptor mRNA (Assay ID Hs00977502_m1) and for Rictor (Assay ID Hs.PT.56a.40621153.g) were purchased from Applied Biosystems and from IDT, respectively. Relative expression was calculated using the comparative cycle threshold method and the endogenous controls human 18S rRNA (Assay ID 4310893E, Applied Biosystems) and RPL13A (Assay ID Hs03043885_g1, Applied Biosystems) as reference genes. Duplicate negative controls (no template cDNA) were also run with every experimental plate to assess specificity and to rule out contamination.

Western blotting

For extract preparation, MTH treated or untreated K562 cells were lysed in a ice cold RIPA buffer (10 mM Tris–HCl, pH 8.0, 0.5 mM EDTA, 150 mM NaCl, 1% NP40, 0.1% SDS, 5 mg/ml DeoxyCholic acid, 1 mM DTT, 2 mM PMSF, 2 mM Na_3VO_4, 10 mM NaF, 1 μg/ml Leupeptin, 1 μg/ml Aprotinin). Briefly, K562 cells (8×10^6 cells) were collected and washed twice with cold PBS (Phosphate-Buffered Saline, Lonza-Biowhittaker, Basel, Switzerland). Cellular pellets were then suspended with 400 μl of cold RIPA buffer, incubated on ice for 20 min and subjected to five cycles of freeze–thawing. Samples were finally centrifuged at 14,000 × *g* for 3 min at 4 °C and the supernatant cytoplasmic fractions were collected and immediately frozen at −80 °C. Protein concentration was determined