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Rapamycin can restore the negative regulatory function of transforming growth factor beta 1 in high grade lymphomas

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ABSTRACT

TGF-β1 (transforming growth factor beta 1) is a negative regulator of lymphocytes, inhibiting proliferation and switching on the apoptotic program in normal lymphoid cells. Lymphoma cells often lose their sensitivity to proapoptotic/anti-proliferative regulators such as TGF-β1. Rapamycin can influence both mTOR (mammalian target of rapamycin) and TGF-β signaling, and through these pathways it is able to enhance TGF-β induced anti-proliferative and apoptotic responses. In the present work we investigated the effect of rapamycin and TGF-β1 combination on cell growth and on TGF-β and mTOR signalling events in lymphoma cells.

Rapamycin, an inhibitor of mTORC1 (mTOR complex 1) did not elicit apoptosis in lymphoma cells; however, the combination of rapamycin with exogenous TGF-β1 induced apoptosis and restored TGF-β1 dependent apoptotic machinery in several lymphoma cell lines with reduced TGF-β sensitivity *in vitro*. In parallel, the phosphorylation of p70 ribosomal S6 kinase (p70S6K) and ribosomal S6 protein, targets of mTORC1, was completely eliminated. Knockdown of Smad signalling by Smad4 siRNA had no influence on apoptosis induced by the rapamycin + TGF-β1, suggesting that this effect is independent of Smad signalling. However, apoptosis induction was dependent on early protein phosphatase 2A (PP2A) activity, and in part on caspases. Rapamycin + TGF-β1 induced apoptosis was not completely eliminated by a caspase inhibitor.

These results suggest that high mTOR activity contributes to TGF-β resistance and lowering mTORC1 kinase activity may provide a tool in high grade B-cell lymphoma therapy by restoring the sensitivity to normally available regulators such as TGF-β1.

1. Introduction

Apoptosis is the default program for B-cells in the absence of survival stimuli. Therefore, the balance between pro- and anti-apoptotic signals are essential for normal B-cell development and function [1]. Early stages of tumorigenesis share similarities with the suppression of apoptotic pathways leading to continuous survival.

Mammalian target of rapamycin complex 1 (mTORC1) lies at the crossroads of signalling networks [2,3], controlled by a wide variety of factors in favour of cell survival. The activation of mTORC1 is an early and frequent event in several tumors [4–6]. Rapamycin (an inhibitor of mTORC1 and a known immunosuppressive agent) and its analogues (rapalogues) inhibit the activity of mTORC1 and the phosphorylation of its downstream targets, p70 ribosomal S6 kinase (p70S6K), ribosomal S6 protein and eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) [7]. The inhibition of mTORC1 yielded therapeutical benefits – such as inhibition of tumor progression and extended overall survival – in recent clinical trials for acute myeloid leukemia (AML), mantle cell lymphoma (MCL), other high grade lymphomas and in non-lymphoid malignancies as well [8–10].

TGF-β1 is a negative regulator of lymphocytes, inhibiting proliferation and switching on the apoptotic program in normal

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lymphoid cells [11]. As expected, TGF-β1 acts as a potent tumour suppressor during the early stages of lymphomagenesis; however, it can promote progression at later stages [12]. The pro-oncogenic function of TGF-β1 signalling may be associated with the loss of TGF-β1 pathway activity and the engagement of other signalling mechanisms, which act in concert during the process of carcinogenesis [13]. While malignant lymphoid cells often lose their sensitivity to proapoptotic/antiproliferative regulators, the failure of the "classical" (Smad-dependent) TGF-β1 pathway is uncommon in lymphoid tumours [14], which raises the importance of "non-classical" (Smad-independent) TGF-β1 signalling as well as the modulatory role of various interacting proteins [15,16]. We have previously shown that exogenous TGF-β1 can induce apoptosis in high grade lymphoma cells in a Smad4-independent and PP2A-dependent manner, primarily through the mitochondrial apoptotic pathway (caspase 9 and 3 activation mediated by FAS, TRAIL and TNF-α receptor independent mitochondrial depolarization) [17,18]. It has been suggested that TGF-β1 resistance could be reversed by lowering the "survival threshold" of cells in certain cases [17].

The interaction between TGF-β1 and mTORC1 signalling and their simultaneous targeting (by TGF-β1 plus rapamycin treatment) has been explored in some cell types, yielding conflicting results [19,20]. It is suggested that rapamycin can modulate TGF-β1 responses by effectively binding to FKBP-12, an inhibitor of TGF-β receptors. The sequestration of FKBP-12 may facilitate TGF-β responses; however, other mechanisms should also be considered.

In the present work we found that inhibition of mTORC1 by rapamycin was able to restore the effectiveness of TGF-β1 to induce apoptosis in high grade B-cell non-Hodgkin lymphomas *in vitro*.

2. Materials and methods

2.1. Cell culture

Experiments were performed on the following human B-cell non-Hodgkin lymphoma (B-NHL) cell lines: HT58 [21] and HT58r (EBV negative cell lines established in our laboratory; HT58r is a subclone of HT58 with low TGF-β1 sensitivity); BL41, BL41/95 (EBV-transfected variant of BL41); Ramos and U266. Cells were cultured in RPMI-1640 (Sigma, St. Louis, MO, USA) with 10% fetal bovine serum (GIBCO-BRL, Grand Island, NY, USA), 0.03% glutamine (, Sigma, St. Louis, MO, USA) and penicillin-streptomycin (100 U/mL–100 μg/mL, Sigma, St. Louis, MO, USA), at 37 °C in 5% CO_2 atmosphere. Cells in the exponential growth phase were used for all experiments.

2.2. Special treatments

Cells (at a density of 1–2 × 10^5/ml) were treated with 1 ng/mL TGF-β1 (reconstituted with 4 mM HCl in 0.1% BSA, aliquoted and stored at −80 °C; R&D Systems, Minneapolis, MN, USA), 50 ng/ml rapamycin (also referred to as low dose – except where indicated otherwise; Sigma, St. Louis, MO, USA), SB431542 TGF-β RI/ALK1 (TGF-β receptor I/activin receptor like kinase-1) 5 μM inhibitor (Sigma, St. Louis, MO, USA), 100 nM okadaic acid (Sigma, St. Louis, MO, USA), LY294002 (5 μM, Calbiochem), Z-VAD-fmk (Sigma, St. Louis, MO, USA, 50 μM) for 0–72 h in 24-well plates or 25 cm^2 flasks. Okadaic acid was used only in the first 4 h of the treatment (short pretreatment), cells were then washed and replated in rapamycin supplemented medium for 72 h. Rapamycin and TGF-β1 were added at the same time in our experiments, as 1–6–12 h pretreatment with either agent showed no significant difference in apoptosis compared to combined treatment (data not shown).

2.3. Cell cycle analysis and apoptosis detection

Apoptosis detection by flow cytometry was performed according to Mihalik et al. [22]. Briefly, cells were fixed in 70% ethanol (−20 °C) followed by alkalic extraction (200 mM Na_2HPO_4, pH 7.4 and 100 μg/ml RNase, Sigma, St. Louis, MO, USA) and ethidium bromide staining (10 μg/ml, Sigma, St. Louis, MO, USA). For each sample, 10,000-20,000 events were acquired using a FACScan flow cytometer (Becton-Dickinson, BD Biosciences, San Diego, CA, USA). Data were analyzed with WinList software (Verity Software House, Topsman, ME, USA). Cell morphology was evaluated on methanol fixed and H&E stained cytospin preparations.

2.4. Knockdown of Smad4 by siRNA

Synthetic Smad4 siRNA (s: r(CAU-CCU-AGU-AAA-UGU-GUUA) dTdT; as: r(UAA-CAC-AUU-UAC-UAG-GAUG)dAdG) (Qiagen GmbH, Hilden, Germany) was used to silence Smad4. A fluorescein labelled negative siRNA control was used as a transfection control. Cells (3 × 10^6) were transfected with 5–10 μl (20 μM) siRNA and 24 μl HiPerFect reagent (Qiagen, Hilden, Germany) in 4 ml medium. Transfection efficiency was determined by flow cytometry after 6–74 h in Smad4 siRNA and fluorescent control siRNA (1:1) co-treated samples and by screening for Smad4 expression and activity. Smad4 expression was detected by Smad4 RT-PCR and Western-blot analysis. Smad4 activity was determined by screening for TIEG (TGF-β induced early gene) mRNA expression in TGF-β1 treated (1–2 h) cultures by RT-PCR. TGF-β1 and rapamycin treatment was initiated 6 h after siRNA transfection.

2.5. RT-PCR

Total RNA was isolated from cells (5–10 × 10^6) with Qiagen RNeasy kit (Qiagen, Hilden, Germany). RNA was reverse transcribed using MMLV Reverse Transcriptase and random primers (Invitrogen, Carlsbad, CA, USA). cDNA (100 ng) was used for PCR. Equal quantity of cDNA was confirmed by β-actin amplification in control and TGF-β1 treated samples by semiquantitative RT-PCR. PCR conditions were as follows: 94 °C 1 min, 55 °C or 60 °C 30 s, 72 °C 45 s; 26–30 cycles using RedTaq polymerase (Sigma, St. Louis, MO, USA). PCR products were resolved by agarose gel (1.5%) electrophoresis, stained with ethidium bromide and analyzed with an Eagle Eye video densitometer (Stratagene, La Jolla, CA, USA). Primers: Smad4 (205 bp, 26–28 cycles) 5′GTG GAA TAG CTC CAG CTA TC3′, 5′CGG CAT GGT ATG AAG TAC TCC3′; TIEG (229 bp, 28 cycles) 5′ACA GGA GAA AAG CCT TTC AGC3′, 5′TTT TAC ATC ACC ACT GGC TCC3′; beta-actin (538 bp, 23-25 cycles) 5′GTG-GGG-CGC-CCC-AGG-CAC-CA3′, 5′CTC-CTT-AAT-GTC-ACG-CAC-GAT-TTC3′.

2.6. Western-blotting

Cells (2 × 10^6) were lysed on ice in sample-buffer (100 μl; containing 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% NP40, 1 mM PMSF, 10 mM NaF, 0.5 mM sodium vanadate, 10 μg/ml leupeptide and 10% glycerol – Sigma, St. Louis, MO, USA; Bio-Rad, Hercules, CA, USA). Lysates were kept on ice for 10 min and centrifuged at 15,000 g for 20 min to collect the supernatant. Protein concentration was measured with the Bradford assay. Equal amounts of protein were diluted with 2xSDS protein sample buffer (60 mM Tris–HCl, 2% SDS, 20% glycerol, 2% β-mercaptoethanol, bromophenolblue; Sigma, St. Louis, MO, USA; Bio-Rad, Hercules, CA, USA), separated on 12.5% SDS-PAGE gels and blotted onto PVDF