HHS Public Access

Author manuscript

Leukemia. Author manuscript; available in PMC 2010 September 16.

Published in final edited form as:

Leukemia. 2009 October ; 23(10): 1867–1874. doi:10.1038/leu.2009.117.

Targeting the mammalian target of Rapamycin to inhibit VEGF and cytokines for the treatment of primary effusion lymphoma

Paola Gasperini[1] and **Giovanna Tosato**[1]

[1]Laboratory of Cellular Oncology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892, USA

Abstract

Primary effusion lymphoma (PEL) is a fatal malignancy, which typically presents as a lymphomatous effusion that later disseminates. Rapamycin (Rapa), which targets mTOR (mammalian target of Rapa), is currently evaluated as a treatment for PEL, but the recent development of PEL in Rapa-treated post-transplant recipients questions the drug's use in PEL. Here, we used a murine model of PEL effusion that mimics the human disease to investigate the anti-PEL activity of Rapa. We found that Rapa reduces ascites accumulation and extends mouse survival. Initially, Rapa reduced PEL load compared to control mice, but most mice rapidly showed PEL progression. Levels of VEGF, which promotes vascular permeability contributing to effusion formation, were significantly reduced in ascites of Rapa-treated mice compared to controls. Expression of IL-10, the principal autocrine growth factor for PEL, was initially reduced in PEL from Rapa-treated mice but rapidly increased despite treatment. We found that the hypoxic environment of ascites and Rapa cooperate in stimulating IL-10 expression in PEL, which likely contributes to the emergence of drug resistance. These results identify Rapa an effective drug to reduce PEL effusions but illustrate the rapid development of drug resistance, which likely limits the efficacy of Rapa in PEL.

Keywords

PEL; Rapamycin; IL-10; KSHV; drug-resistance

Introduction

Primary effusion lymphoma (PEL) is a highly aggressive lymphoma that characteristically presents as an effusion malignancy in the body cavities of patients with AIDS and occasionally HIV-negative patients (1, 2). Typically, PEL is latently infected with Kaposi's sarcoma herpesvirus (KSHV) and is often co-infected with Epstein-Barr virus (1, 2). In the setting of AIDS, the clinical course of most of these lymphomas is generally aggressive,

Correspondence: Dr. Paola Gasperini, Laboratory of Cellular Oncology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, 37 Convent Drive 37/4, Bethesda, MD 20892, USA; gasperinip@mail.nih.gov; Phone: (301) 594-9591; Fax: (301) 594-9585.

Supplementary information is available at Leukemia@leukemianature.com

with a mean survival from diagnosis of 5–7 months despite chemotherapy (3). Thus, there is a need for improved therapy.

Rapa (sirolimus), a macrolide compound approved as an immunosuppressant drug, was shown to exert antineoplastic and antiangiogenic properties (4, 5). Rapa complexes with the protein FK506-binding protein 12, which functionally inhibits the mammalian target of Rapa (mTOR) (6). mTOR is a regulatory kinase that controls fundamental cell functions, including translation initiation, transcription of stress response genes, ribosomal biogenesis and tRNA synthesis, and thus plays a central role in cell cycle progression, cell growth and survival (7). As an inhibitor of mTOR, Rapa inhibits the growth of many cancer cell types in vitro (8). Stallone et al. reported that switching from cyclosporine-A to Rapa for immunosuppression led to regression of Kaposi's sarcoma (KS) in 15 kidney transplant recipients (9). Since KS and PEL are both KSHV-infected, this observation prompted an evaluation of the potential utility of Rapa in PEL. A recent study reported that Rapa was effective at preventing/delaying development of subcutaneous (sc) PEL tumors in mice, leading to the investigational use of Rapa in patients with PEL (10). More recently, two HIV-1-negative patients developed PEL while receiving Rapa for immunosuppression following kidney transplantation (11), raising important questions on the safety and efficacy of Rapa in patients with PEL.

To address these questions, we exploited a murine model of PEL that mimics the human disease in being a peritoneal effusion lymphoma that subsequently progresses to form solid tumors. We have found that Rapa induces an initial response but does not eradicate PEL. With continued treatment, environmental stress from the hypoxic environment of the peritoneal cavity initiates a cytokine-mediated escape mechanism leading to disease progression.

Materials and Methods

Cell lines

PEL cell lines BC-1 (from Dr. Y. Chang, Univ. of Pittsburg, PA), BCBL-1 and JSC-1 (from Dr. R. Yarchoan, NCI, Bethesda, MD), BC-3 (ATCC, Rockville MD) and VG-1 (from Dr. David Scadden, Harvard Medical School, Boston, MA, USA) were maintained in RPMI1640 (GIBCO, Invitrogen, Carlsbard, CA) with 10% heat-inactivated fetal bovine serum (FBS), 1mM glutamine, at 37°C in 5% CO_2. All these cell lines are KSHV-positive; BC-1and JSC-1 cells are also EBV-positive.

Establishment of PEL ascites and tumors

Animal experiments were approved by the NCI Animal Care and Use Committee. Female NOD/SCID mice (6-week old) were injected intraperitoneally (ip) with BC-1 cells (2×10^7 cells/mouse in 0.2 ml PBS). Groups of mice were either observed untreated or received daily ip injections of Rapa (Sigma-Aldrich, [St. Louis, MO] 3mg/kg, in 0.2 ml diluent) or diluent only, beginning 24-h after PEL inoculation until sacrifice. This Rapa dose was selected based on previous experiments (4). Animals were euthanized (per protocol) when they

developed excessive abdominal distension interfering with movement or causing other distress.

Immunoblotting

Protein extracts for phosphorylated proteins prepared in SDS lysis buffer with protease inhibitor cocktail setIII (Calbiochem, Darmstadt, Germany), 50 mM NaF, and 1 mM sodium orthovanadate were resolved in NuPAGE 4-12% Bis-Tris Gel (Invitrogen), transferred to protran membranes (Whatman GmbH, Dassel, Germany). Immunoblotting antibodies for actin, phospho-S6 ribosomal protein (Ser235/236), S6 ribosomal protein, phospho-Akt (Ser473), phospho-Akt (Thr308), Akt, phospho-GSK-3beta (ser9), GSK-3beta, phospho-Src family (Tyr416), non-phospho-Src, phospho-eNOS (ser1177) and eNOS were from Cell Signaling Technologies (Beverly, MA); antibody for LANA was from ABI (Columbia, MD). Monoclonal antibody to RTA was a gift of Dr. Kazu (NCI, Bethesda, MD); rabbit polyclonal antibody to vFLIP was a gift of Dr. P. Chaudhary (Hillman Cancer Center, Pittsburg, PA). Monoclonal antibodies for vIL-6 (clone 12.1.1) were from our laboratory (12). Bound secondary antibodies were visualized by enhanced chemiluminescence (Amersham, Arlington Heights, IL).

Cell proliferation assays

PEL cells (5×10^3-4×10^4 cells/well, 96-well plates) were incubated in RPMI1640 with 10% FBS or with 0.25mg/ml BSA plus 0.25mM sodium pyruvate, 5μM HEPES and 2.5μg/ml transferrin for 72h at 37°C with or without Rapa. Proliferation was measured by 6-h pulse with 1 μCi/well [^{3}H]-thymidine (Amersham).

Immunohistochemistry

Tumor sections were deparaffinized, rehydrated, boiled in 10mM sodium citrate buffer (pH 6.0), cooled, blocked for 1 h at RT (TBS with 10% horse serum [Biosorce, Camarillo, CA] and 5% BSA), and incubated overnight (4°C) with phospho-S6 ribosomal protein (Ser235/236) antibody, rabbit monoclonal S6 ribosomal protein antibody (Cell Signaling Technology). Sections were stained with a goat anti-rabbit biotinylated horseradish peroxidase H-conjugated secondary antibody followed by Avidin DH (VECTASTAIN ABC kit; Vector Laboratories) and by Vector *Nova* Red substrate for peroxidase (Vector Laboratories, Burlingame, CA). Slides were counterstained with Accustain (Sigma). Images were recorded using Olympus BX41 microscope (Olympus, Center Valley, PA).

Cytokines measurements

Human (h)IL-10, hIL-6, human VEGF levels were measured using enzyme-linked immunoabsorbent assay (Quantikine, R&D Systems, Minneapolis, MN). The ELISA for detection of vIL-6 was described (12). Cytokine mRNA levels were measured by real time PCR (Supplementary Materials and Methods) with primers for hIL-10 and hIL6 from Applied Biosystems (Foster City, CA); primers for vIL-6 were: 5′-ACG CGG GGC AAG TTG CCG GAC-3′ (forward) and 5′-TAC TTA TCG TGG ACG TCA GGA-3′ (reverse).

Statistical analysis

Statistical significance of group differences was evaluated by Student *t* test, Fisher's exact test, Wilcoxon rank sum test, and Kruskal-Wallis test.

Results

Rapa inhibits PEL cell proliferation in vitro

We examined whether mTOR is constitutively active in PEL cells by evaluating the phosphorylation status of the mTOR effector S6. We found that S6 is constitutively phosphorylated in the PEL cell lines BC-1, BCBL-1, BC-3, JSC-1 and VG-1, indicative of constitutive mTOR activity in culture. Incubation with 100nM Rapa for 72h consistently reduced such phosphorylation (Fig. 1A). We examined the effect of Rapa on PEL cell growth (bar graph, Fig. 1B). Rapa reduced proliferation in all cell lines tested. VG-1 cells proved the most sensitive (92% reduction with 10nM Rapa) and BCBL-1 cells proved the least sensitive (26% reduction with 1000nM Rapa). We evaluated cell viability in these cultures. At 1000nM, Rapa reduced cell viability to 87.7% in BC-1 cells but minimally affected viability of all other cell lines (line graph, Fig. 1B). We conclude that Rapa exerts a variable cytostatic effect on PEL cells cultured in vitro, but has little cytotoxicity for these cells.

Rapa inhibits accumulation of PEL ascites

We used a mouse model of PEL to evaluate the anti-tumor effects of Rapa in vivo. PEL cells are inoculated intraperitoneally (ip) in NOD/SCID mice resulting in the development of PEL ascites and subsequent formation of solid tumors arising from the parietal and/or visceral mesothelial layer of the peritoneum. This model closely resembles human PEL in displaying a body cavity location, development of lymphomatous ascites and solid body-cavity lymphoma (1, 2). Pilot experiments showed that PEL cell lines display varying degree of sensitivity to Rapa treatment in vivo (not shown). We selected the BC-1 cell line for further investigation because it displayed an intermediate sensitivity to Rapa. 20×10^6 BC-1 cells were injected ip into 15 NOD/SCID mice; 3 mice were observed untreated, and 12 mice were treated with Rapa (3 mg/kg/mouse/day ip) starting 1 day after BC-1 cell injection. By day 15, all control mice developed massive PEL ascites necessitating sacrifice per protocol. Three Rapa-treated mice with no evidence of disease were also electively sacrificed on day 15. The remaining mice were maintained on Rapa until day 25, when they were electively sacrificed. Rapa significantly ($P<0.001$) extended the survival of PEL-bearing mice (Fig. 2A).

We evaluated in all mice a) ascites volume; b) PEL load in the ascitic fluid; and c) presence of tumors at autopsy. All control mice and 10/12 Rapa-treated mice (including 3/3 mice sacrificed on day 15 and 7/9 mice sacrificed on day 25) had ascites, but the mean volume was significantly ($p<0.01$) reduced in the Rapa-treated mice (Fig. 2B, left panel). The volume of cell-free ascites from the Rapa-treated mice was reduced by 95% in mice sacrificed on day 15 and by 88% in mice sacrificed on day 25 compared to the untreated mice (Fig. 2B, mid panel). We counted PEL cells recovered from all ascites (Fig. 2B, right panel). In the controls, we recovered significantly ($p=0.014$) more PEL cells than from the

treated animals (65 and 46% reduction on day 15 and 25, respectively). The 2 Rapa-treated mice sacrificed on day 25 without ascites at autopsy had PEL cells in the peritoneal lavage, but fewer cells were recovered than originally injected. The 10 remaining mice had more PEL cells in the ascites than originally injected, evidence that PEL cells had proliferated in vivo despite treatment. PEL cell viability from Rapa-treated mice (75.55±14.7%) was similar to that from control mice (81.0%±3.9). These results indicate that Rapa reduced PEL progression predominantly by reducing fluid accumulation and PEL growth rather than by promoting PEL cell death.

Only 2/12 Rapa-treated mice displayed a solid tumor mass on autopsy, a frequency that was lower than that observed in 17 non parallel control mice injected with BC-1 cells on the same protocol and were sacrificed 18-24 days post PEL injection (2/12 versus 10/17: P=0.054 Fisher's exact test). The size of the 2 tumors recovered from the Rapa-treated mice (0.24 and 0.1 cm^3, mean=0.17) was smaller than that from the control mice (5.9±5.3cm^3), but the difference was not significant (p=0.076, exact Wilcoxon rank sum test). Thus, treatment with Rapa extended the survival of PEL-bearing mice, reduced accumulation of ascites and limited PEL cell growth, but did not clear the mice of PEL.

Rapa blocks mTOR activity *in vivo*

The downstream effector of mTOR, S6 is costitutively phosphorylated in cultured PEL cells, and Rapa reduces this constitutive phosphorylation in vitro (Fig. 1A). We examined the phosphorylation status of S6 in PEL cells from the mice. S6 protein was phosphorylated to a lower degree in PEL cells recovered Rapa-treated animals (from day 15 and day 25) compared to controls, providing biochemical evidence of drug activity in vivo (Fig. 2C). By immunohistochemistry, we detected phosphorylated S6 protein in PEL tumors from untreated mice (representative results in Fig. 2D), which was reduced in the only 2 PEL tumors recovered from treated mice (Fig. 2D). Thus, PEL progression on Rapa occurred in spite of biochemical evidence of persistent drug activity.

Rapa inhibits VEGF secretion and signaling

Malignant ascites results from increased peritoneal capillary permeability, accumulation of plasma exudates in the cavity, and reduced drainage by tumor-infiltrated diaphragmatic lymphatic vessels (13). This process is driven by vascular endothelial growth factor (VEGF) (13). Our group has previously demonstrated that VEGF neutralization prevents accumulation of PEL ascites (14). Since Rapa reduces accumulation of ascites (Fig. 2B), we hypothesized that Rapa reduces VEGF secretion and/or responses to VEGF. We found that Rapa (100nM) minimally reduces VEGF secretion in BC-1 cells over 72h culture (results expressed/10^6cells, Fig. 3A). Nonetheless, VEGF concentration in the culture supernatants (results expressed/ml) was significantly lower after incubation with Rapa compared to medium only (p=0.006) due to reduced cell growth. VEGF mRNA levels were minimally affected by Rapa (100nM; 72 hr) in BC-1 cells (Fig. 3B). However, we found that 15 and 25 days Rapa treatment reduced significantly hVEGF secretion by PEL (results expressed/10^6cells, Fig. 3C). This reduction was not accompanied by a reduction in the levels of VEGF mRNA (Fig. 3D), and is likely attributable to reduced VEGF translation after prolonged exposure to the drug. Murine (m) VEGF, which was measurable in ascites at

concentrations ~50-fold lower than those of human (h, tumor-derived) VEGF, was similarly reduced by Rapa treatment (not shown). Thus, treatment of mice with Rapa significantly reduces hVEGF secretion by PEL cells and mVEGF by the host cells.

Rapa exerts anti-angiogenic effects by decreasing VEGF production and blocking Akt/mTOR signaling in VEGF-activated endothelial cells (15). We tested the effects of Rapa on VEGF-induced signaling by incubating HUVEC with 100nM Rapa for 72h and then adding VEGF (100ng/ml) for 30 minutes. We found that Rapa reduces VEGF-induced activation of Akt (Ser473 and Thr308) and the Akt/mTOR effectors eNOS, GSK3 beta, P70s6K and S6 (Fig. 3E). We conclude that Rapa can reduce both VEGF production by PEL and VEGF responses by endothelial cells, including eNOS activation, which has been prominently linked to the permeability-promoting effects of VEGF (16-19). These results explain the marked reduction of ascites accumulation in Rapa-treated mice bearing PEL.

Effects of Rapa on expression of autocrine growth factors

PEL cells secrete IL-10, IL-6 and vIL-6 (20-22), and utilize IL-10 and vIL-6, but not IL-6, as autocrine growth factors (23). Rapa was reported to variably reduce PEL secretion of these proteins (10, 22); we wished to confirm these results. BC-1 (5×10^5/ml) cells were cultured (72h) in medium alone or with 100nM Rapa; cells were counted and concentrations of IL-10, IL-6 and vIL-6 measured by ELISA in the supernatants. Since Rapa reduces BC-1 cell proliferation (Fig. 1B), cytokine levels are expressed relative to cell number (ng/10^6). Secretion of IL-10 and IL-6, but not vIL-6, was significantly reduced by Rapa (Fig. 4A). Since IL-10 is the most potent autocrine growth factor for PEL, reduced IL-10 secretion likely contributes to reduced PEL cells proliferation with Rapa.

To examine whether Rapa treatment in vivo reduces cytokine secretion, we measured IL-10, IL-6 and vIL-6 concentrations in the cell-free ascites from PEL-bearing mice. Since treatment with Rapa reduced PEL cell recovery from ascites, the results are expressed relative to PEL cell number. We found human (h) IL-10 (human-specific ELISA), vIL-6 and hIL-6 (human-specific ELISA) levels to be significantly reduced in the ascites from mice treated with Rapa for 15 days compared to controls (Fig 4B). The reduction of vIL-6 and hIL-6 continued to be significant in mice treated with Rapa for 25 days. However, levels of hIL-10 were no longer reduced in the ascites of mice treated for 25 days with Rapa compared to controls. This was surprising because S6 phosphorylation continued to be reduced in PEL cells recovered after 25-days treatment with Rapa (Fig. 2C), indicative of persisting drug activity.

We investigated potential mechanisms underlying the rise in IL-10 secretion by PEL after prolonged treatment with Rapa. Short-term (72 hr) exposure to Rapa in vitro minimally altered mRNA levels of IL-10, vIL-6 and IL-6 in PEL cells (Fig. 4C); Rapa treatment in vivo for 15 and 25 days did not significantly alter levels of hIL-6 mRNA in PEL cells recovered from ascites. Instead, levels of hIL-10 and vIL-6 mRNAs were significantly increased in PEL cells from mice treated with Rapa for 15 and 25 days (Fig. 4D). vIL-6 mRNA expression doubled on Rapa treatment (15 and 25-days), but this increase was not accompanied by an increase of vIL6 in the ascites (Fig. 4B), possibly due to effective translational block by Rapa. Levels of hIL-10 mRNA increased two-fold in PEL from 15-

day-treated and six-fold from 25-day-treated mice compared to controls (Fig. 4D). This progressive increase in hIL-10 mRNA levels likely underlies the progressive increase of hIL-10 in the ascites of treated mice. Interestingly, hIL-10 mRNA levels in PEL cells from the peritoneal lavage of the only two mice without ascites after 25-day treatment with Rapa were not increased compared to untreated controls ($P>0.05$), suggesting that increased IL-10 transcription is critical to the survival/proliferation of PEL cells and may be a surrogate marker of Rapa activity in PEL.

Effects of hypoxia on IL-10 expression and sensitivity to Rapa

To investigate mechanisms for increased IL-10 expression in PEL from mice on prolonged Rapa treatment, we tested whether stress from the hypoxic environment of ascites (24, 25) contributes to increased IL-10 expression in PEL cells. BC-1 cells (5×10^5/ml) were incubated for 72h under hypoxic (1% O2, 5% CO2, 94% N2) or normoxic conditions with or without 100nM Rapa; levels of IL-10 protein and mRNA were measured in cells and supernatants, respectively. Consistent with the results in Fig. 4A, we found that Rapa reduces IL-10 levels in the culture supernatants of BC-1 cells cultured under normoxic conditions (Fig. 5A), but did not decrease IL-10 secretion in BC-1 cells cultured under hypoxic conditions (Fig. 5A). Rapa had similar effects on IL-10 secretion in BC-3 cells (not shown). Rapa did not alter IL-10 mRNA levels in BC-1 cells cultured under normoxic conditions, but promoted a 3-fold increase in IL-10 mRNA levels in BC-1 cells cultured under hypoxic conditions (Fig. 5B). Rapa did not enhance IL-6 mRNA levels under hypoxic conditions (not shown).

To test whether PEL cells maintain responsiveness to IL-10 on Rapa, BC-1 cells were cultured (72h) with IL-10 (2-500 pg/ml) with or without Rapa. Without Rapa, BC-1 cells dose-dependently proliferated to IL-10, but not IL-6 (not shown), displaying maximal responses at 500 pg/ml (Fig. 5C). With Rapa, BC-1 cells continued to dose-dependently respond to IL-10 (Fig. 5D), consistent with results (not shown) indicating that Rapa does not alter STAT1 and STAT3 signaling in BC-1 cells. Similar results were obtained using BC-3 cells (not shown). The results shown in Fig. 5A and B show that the hypoxic environment in ascites likely contributes to promote IL-10 expression in PEL cells from mice treated with Rapa, and provide evidence that increased IL-10 expression represents a mechanism for resistance to the cytostatic effects of Rapa.

Rapa does not promote KSHV replication

Since KSHV infection is a defining feature of PEL and Rapa inhibits vIL-6 expression in mice treated with the drug (Fig. 4B), we explored more broadly the effects of Rapa on latent, early lytic and late lytic and viral gene expression. We found that Rapa minimally alters mRNA and protein levels of ORF73/LANA, K13/vFLIP, ORFK2/vIL-6, ORK50/RTA and ORF8.1/gpK8.1 (Supplementary Fig.1). These results demonstrate that Rapa does not promote KSHV replication in PEL cells and that its effects on PEL are unrelated to change in the virus life cycle.

Discussion

Exciting preclinical results showing that Rapa is effective at preventing formation of sc PEL tumors in mice raised the possibility that Rapa may represent a new treatment option for patients with PEL (10). This enthusiasm was recently tempered by a report describing the development of PEL in two HIV-negative kidney transplant recipients receiving Rapa for immunosuppression (11). Rapa has been used extensively for immunosuppression to prevent rejection of kidney transplants, and has shown anti-tumor activity against some tumors, including KS (5). Both KS and PEL are infected with KSHV in AIDS patients and may co-exist. Here, we selected a murine model of PEL that mimics human PEL in its characteristic location and mode of progression to re-evaluate the effectiveness of Rapa against PEL. We found that Rapa strikingly reduces the accumulation of ascites, delays PEL progression and extends mouse survival, but does not eradicate the disease. Increased IL-10 expression by PEL on extended Rapa treatment contributes to drug resistance.

Analysis of the mechanisms for the striking reduction of ascitic fluid accumulation induced by Rapa revealed that the drug inhibits VEGF secretion by PEL cells and the mouse host, and limits endothelial cell responses to VEGF. It is now clear that VEGF, which promotes vascular permeability, plays a critical role in the formation of neoplastic effusions (26, 27). Here, we found that Rapa not only post-transcriptionally reduces VEGF production but also blocks VEGF-induced signaling in endothelial cells, including activation of P-Akt (ser473) and P-eNOS, which are critical mediators of VEGF-induced vascular permeability (15, 28). These results are consistent with previous results showing that Rapa inhibits constitutive Akt activation in endothelial cells (15) and that Akt activation is necessary for VEGF permeability (28), suggesting that Rapa could be effective at reducing formation of edema and effusions (15, 29). The current results provide strong evidence that Rapa is an effective inhibitor of body fluids accumulation, which is of potential clinical importance.

Rapa did not eradicate PEL because the drug is not cytotoxic for PEL. After an initial response, PEL cells develop resistance to Rapa. Consistent with previous observations (10, 30, 31), we found that Rapa post-transcriptionally down-regulates PEL production of IL-10 but does not alter IL-10 signaling pathways. Since IL-10 is the principal autocrine growth factor for PEL (23), reduced IL-10 secretion is likely responsible for decreased PEL cell proliferation with Rapa. The resistance to Rapa that rapidly ensues is attributable, at least in part, to increased IL-10 expression on Rapa treatment. We could reproduce in culture key features of this evolution. Rapa was no longer effective at reducing IL-10 secretion by PEL cells exposed to hypoxic stress, a feature of body cavity effusions (24, 25). Rather, under hypoxic conditions, Rapa promoted IL-10 mRNA expression in PEL cells and enhanced PEL cell survival/proliferation. Thus, we conclude that PEL is only transiently responsive to Rapa because PEL develop an escape mechanism resulting in increased production of IL-10, which promotes PEL cell growth. The only 2 mice that exhibited a persistent response to Rapa harbored PEL cells that did not show increased IL-10 expression. This suggests that stress factors active in the microenvironment of lymphomatous effusions are important inducers of Rapa resistance in PEL.

There is enthusiasm for the development of Rapa and other mTOR inhibitors as cancer therapeutics. Cytotoxicity is generally viewed as essential to effective anticancer therapy, but we find that Rapa is mostly cytostatic for PEL cell lines. It is possible that Rapa is cytotoxic for primary PEL and not for PEL cell lines, which may have developed resistance to the drug through acquired genetic mutations. If primary PEL cells and PEL cell lines respond similarly to Rapa, dual inhibition of mTOR and IL-10 function could be an effective anti-PEL strategy since increased IL-10 expression appears critical to PEL cell lines resistance to Rapa. Importantly, the current results present strong evidence supporting the investigational use of Rapa to reduce fluid accumulation in body cavities affected with PEL and potentially other types of malignant effusions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Supported by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research. P.G. supported in part by F.I.R.C. (Italian Foundation for Cancer Research). We thank Drs. S. Steinberg, K. Ueda, R. Yarchoan, S. Pittaluga, D. Whitby, S. Sakakibara, M. Segarra and P. McCormick for help on aspects of this work.

References

1. Cesarman E, Chang Y, Moore PS, Said JW, Knowles DM. Kaposi's sarcoma-associated herpesvirus-like DNA sequences in AIDS- related body-cavity-based lymphomas. N Engl J Med. 1995; 332(18):1186–1191. [PubMed: 7700311]
2. Nador RG, Cesarman E, Chadburn A, Dawson DB, Ansari MQ, Sald J, et al. Primary effusion lymphoma: a distinct clinicopathologic entity associated with the Kaposi's sarcoma-associated herpes virus. Blood. 1996; 88(2):645–656. [PubMed: 8695812]
3. Simonelli C, Spina M, Cinelli R, Talamini R, Tedeschi R, Gloghini A, et al. Clinical features and outcome of primary effusion lymphoma in HIV-infected patients: a single-institution study. J Clin Oncol. 2003 Nov 1; 21(21):3948–3954. [PubMed: 14581418]
4. Guba M, von Breitenbuch P, Steinbauer M, Koehl G, Flegel S, Hornung M, et al. Rapamycin inhibits primary and metastatic tumor growth by antiangiogenesis: involvement of vascular endothelial growth factor. Nat Med. 2002 Feb; 8(2):128–135. [PubMed: 11821896]
5. Guertin DA, Sabatini DM. Defining the Role of mTOR in Cancer. Cancer Cell. 2007 Jul; 12(1):9–22. [PubMed: 17613433]
6. Wullschleger S, Loewith R, Hall MN. TOR signaling in growth and metabolism. Cell. 2006 Feb 10; 124(3):471–484. [PubMed: 16469695]
7. Sarbassov DD, Ali SM, Sabatini DM. Growing roles for the mTOR pathway. Curr Opin Cell Biol. 2005 Dec; 17(6):596–603. [PubMed: 16226444]
8. Huang S, Houghton PJ. Inhibitors of mammalian target of rapamycin as novel antitumor agents: from bench to clinic. Curr Opin Investig Drugs. 2002 Feb; 3(2):295–304.
9. Stallone G, Schena A, Infante B, Di Paolo S, Loverre A, Maggio G, et al. Sirolimus for Kaposi's sarcoma in renal-transplant recipients. N Engl J Med. 2005 Mar 31; 352(13):1317–1323. [PubMed: 15800227]
10. Sin SH, Roy D, Wang L, Staudt MR, Fakhari FD, Patel DD, et al. Rapamycin is efficacious against primary effusion lymphoma (PEL) cell lines in vivo by inhibiting autocrine signaling. Blood. 2007 Mar 1; 109(5):2165–2173. [PubMed: 17082322]

11. Boulanger E, Afonso PV, Yahiaoui Y, Adle-Biassette H, Gabarre J, Agbalika F. Human herpesvirus-8 (HHV-8)-associated primary effusion lymphoma in two renal transplant recipients receiving rapamycin. Am J Transplant. 2008 Mar; 8(3):707–710. [PubMed: 18261181]
12. Aoki Y, Narazaki M, Kishimoto T, Tosato G. Receptor engagement by viral interleukin-6 encoded by Kaposi sarcoma-associated herpesvirus. Blood. 2001; 98(10):3042–3049. [PubMed: 11698289]
13. Nagy JA, Morgan ES, Herzberg KT, Manseau EJ, Dvorak AM, Dvorak HF. Pathogenesis of ascites tumor growth: angiogenesis, vascular remodeling, and stroma formation in the peritoneal lining. Cancer Res. 1995; 55(2):376–385. [PubMed: 7529135]
14. Aoki Y, Tosato G. Role of vascular endothelial growth factor/vascular permeability factor in the pathogenesis of Kaposi's sarcoma-associated herpesvirus-infected primary effusion lymphomas. Blood. 1999; 94(12):4247–4254. [PubMed: 10590069]
15. Phung TL, Ziv K, Dabydeen D, Eyiah-Mensah G, Riveros M, Perruzzi C, et al. Pathological angiogenesis is induced by sustained Akt signaling and inhibited by rapamycin. Cancer Cell. 2006 Aug; 10(2):159–170. [PubMed: 16904613]
16. Bucci M, Roviezzo F, Posadas I, Yu J, Parente L, Sessa WC, et al. Endothelial nitric oxide synthase activation is critical for vascular leakage during acute inflammation in vivo. Proc Natl Acad Sci U S A. 2005 Jan 18; 102(3):904–908. [PubMed: 15640348]
17. Fukumura D, Gohongi T, Kadambi A, Izumi Y, Ang J, Yun CO, et al. Predominant role of endothelial nitric oxide synthase in vascular endothelial growth factor-induced angiogenesis and vascular permeability. Proc Natl Acad Sci U S A. 2001 Feb 27; 98(5):2604–2609. [PubMed: 11226286]
18. Hatakeyama T, Pappas PJ, Hobson RW 2nd, Boric MP, Sessa WC, Duran WN. Endothelial nitric oxide synthase regulates microvascular hyperpermeability in vivo. J Physiol. 2006 Jul 1; 574(Pt 1):275–281. [PubMed: 16675496]
19. Eliceiri BP, Paul R, Schwartzberg PL, Hood JD, Leng J, Cheresh DA. Selective requirement for Src kinases during VEGF-induced angiogenesis and vascular permeability. Mol Cell. 1999 Dec; 4(6):915–924. [PubMed: 10635317]
20. Jones D, Ballestas ME, Kaye KM, Gulizia JM, Winters GL, Fletcher J, et al. Primary-effusion lymphoma and Kaposi's sarcoma in a cardiac-transplant recipient [see comments]. N Engl J Med. 1998; 339(7):444–449. [PubMed: 9700178]
21. Aoki Y, Jaffe ES, Chang Y, Jones K, Teruya-Feldstein J, Moore PS, et al. Angiogenesis and hematopoiesis induced by Kaposi's sarcoma-associated herpesvirus-encoded interleukin-6. Blood. 1999 Jun 15; 93(12):4034–4043. [PubMed: 10361100]
22. Foussat A, Balabanian K, Amara A, Bouchet-Delbos L, Durand-Gasselin I, Baleux F, et al. Production of stromal cell-derived factor 1 by mesothelial cells and effects of this chemokine on peritoneal B lymphocytes. Eur J Immunol. 2001 Feb; 31(2):350–359. [PubMed: 11180098]
23. Jones KD, Aoki Y, Chang Y, Moore PS, Yarchoan R, Tosato G. Involvement of interleukin-10 (IL-10) and viral IL-6 in the spontaneous growth of Kaposi's sarcoma herpesvirus-associated infected primary effusion lymphoma cells. Blood. 1999; 94(8):2871–2879. [PubMed: 10515891]
24. Funahashi A, Sarkar TK, Kory RC. PO 2, PCO 2, and pH in pleural effusion. J Lab Clin Med. 1971 Dec.78(6):1006. [PubMed: 5131833]
25. Houston MC. Pleural effusion: diagnostic value of measurements of PO2, PCO2, and pH. South Med J. 1981 May; 74(5):585–589. [PubMed: 6787716]
26. Luo JC, Yamaguchi S, Shinkai A, Shitara K, Shibuya M. Significant expression of vascular endothelial growth factor/vascular permeability factor in mouse ascites tumors. Cancer Res. 1998; 58(12):2652–2660. [PubMed: 9635593]
27. Mesiano S, Ferrara N, Jaffe RB. Role of vascular endothelial growth factor in ovarian cancer: inhibition of ascites formation by immunoneutralization. Am J Pathol. 1998; 153(4):1249–1256. [PubMed: 9777956]
28. Gerber HP, McMurtrey A, Kowalski J, Yan M, Keyt BA, Dixit V, et al. Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3′-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. J Biol Chem. 1998 Nov 13; 273(46):30336–30343. [PubMed: 9804796]

29. Mabuchi S, Altomare DA, Connolly DC, Klein-Szanto A, Litwin S, Hoelzle MK, et al. RAD001 (Everolimus) delays tumor onset and progression in a transgenic mouse model of ovarian cancer. Cancer Res. 2007 Mar 15; 67(6):2408–2413. [PubMed: 17363557]

30. Nepomuceno RR, Balatoni CE, Natkunam Y, Snow AL, Krams SM, Martinez OM. Rapamycin inhibits the interleukin 10 signal transduction pathway and the growth of Epstein Barr virus B-cell lymphomas. Cancer Res. 2003 Aug 1; 63(15):4472–4480. [PubMed: 12907620]

31. Vaysberg M, Balatoni CE, Nepomuceno RR, Krams SM, Martinez OM. Rapamycin inhibits proliferation of Epstein-Barr virus-positive B-cell lymphomas through modulation of cell-cycle protein expression. Transplantation. 2007 Apr 27; 83(8):1114–1121. [PubMed: 17452903]

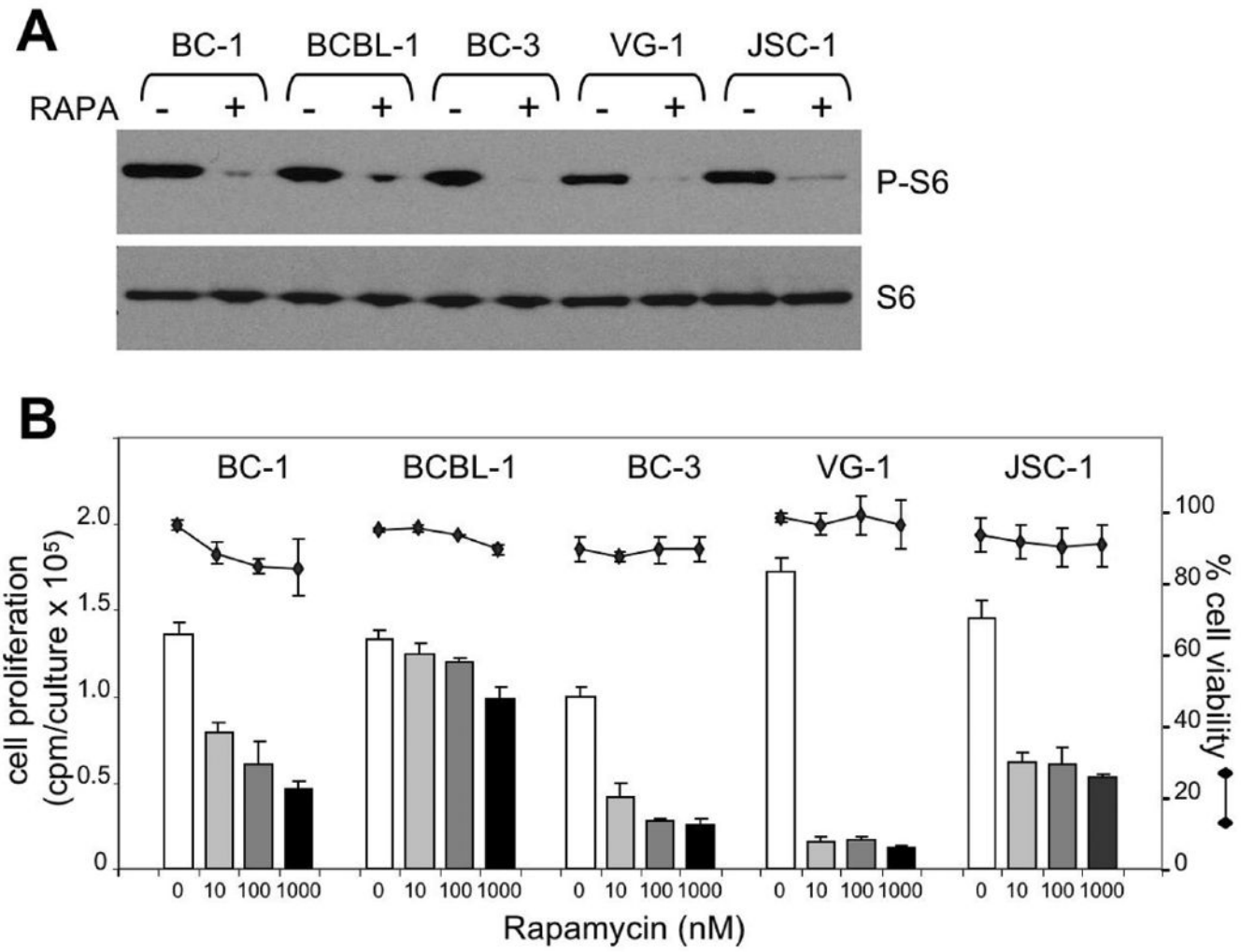

Figure 1. Effects of Rapa on signaling mediators, PEL cell growth and survival

(A) PEL cells from 5 PEL cell lines were incubated for 72h, in maintenance medium only (RPMI 1640 supplemented with 10%FBS) or with 100nM Rapa. Cell lysates were immunoblotted with specific antibodies. The results reflect probing and stripping of a single membrane. **(B)** Effects of Rapa on PEL cell proliferation and viability. PEL cells were cultured (5×10^4 cells/ml; 37°C) in medium only (RPMI 1640 with 10% FBS) or with addition of Rapa (10-1000nM). Proliferation (bar-graph) was measured by ^{3}H thymidine incorporation during the final 6h of a 3-day culture; viability (line-graph) was evaluated by flow cytometry after 15 minutes incubation with propidium iodide. The results reflect the means (±SE) of triplicate cultures (representative results from 5 experiments).

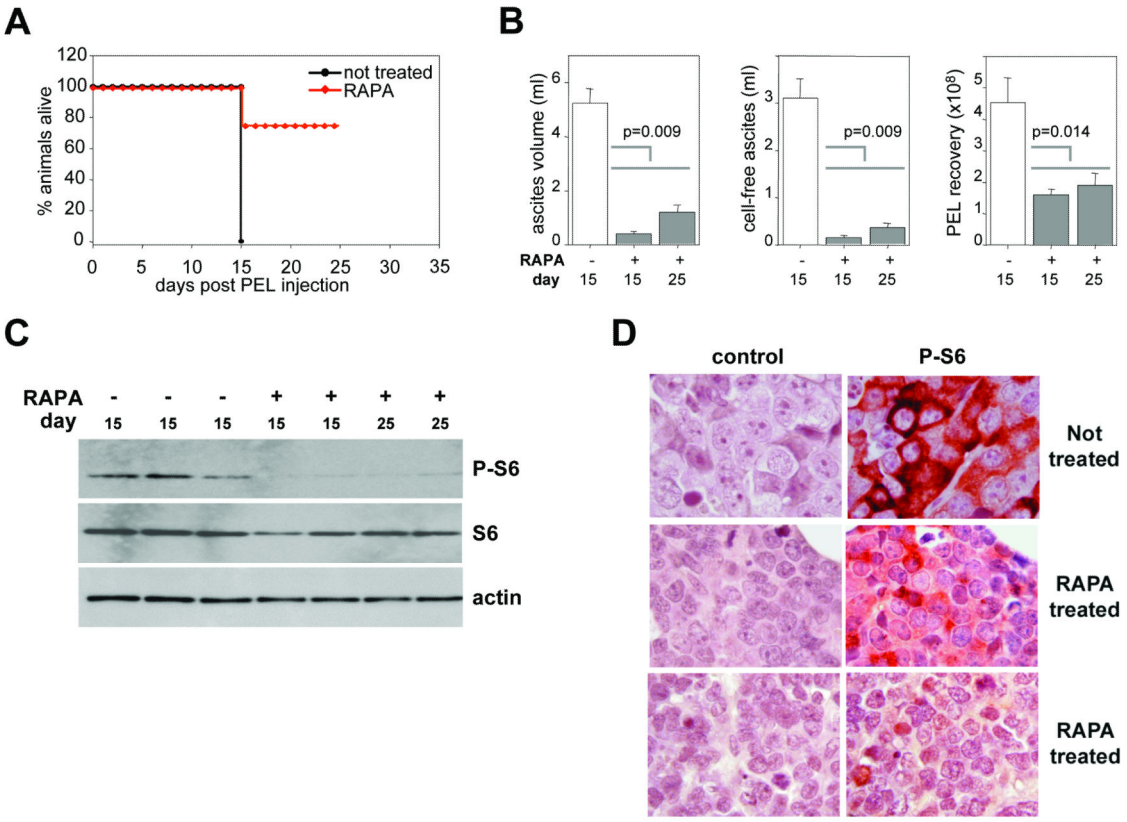

Figure 2. Effects of Rapa treatment on experimental PEL

(A) NOD/SCID mice inoculated ip with BC-1 cells (20×10^6) were either observed untreated (3 mice) or treated daily with ip Rapa (12 mice; 3 mg/kg/day). All control mice had to be sacrificed on day 15 due to excessive ascites; 3 Rapa-treated mice were electively sacrificed on day 15; the remainder 9 mice were continued on Rapa for 10 days at which time they were electively sacrificed. The results reflect % survival as a function of days post PEL cell inoculation. **(B)** Ascites volume including (left panel) and excluding (middle panel) PEL cell pellet, and PEL cells recovered (right panel) from ascites of control mice and Rapa-treated mice (mice sacrificed on day 15 and on day 25). The results reflect the means (±SE) of all treated mice that developed ascites. Statistical significance of group differences is shown. **(C)** Cell lysates of BC-1 cells recovered from the ascites of 3 controls (no Rapa), 2 Rapa-treated mice sacrified on day 15 and on day 25 were immunoblotted with specific antibodies. The results reflect probing and stripping of a single membrane. (**D**) Microscopic histology and immunohistochemical analysis of P-S6 expression in tumor tissues from a representative control mouse and from 2 Rapa-treated mice that developed a peritoneal tumor mass. Control staining and P-S6 immunostating are shown for each tumor; images from each tumor reflect the same field from sequential sections. Original magnification ×100.

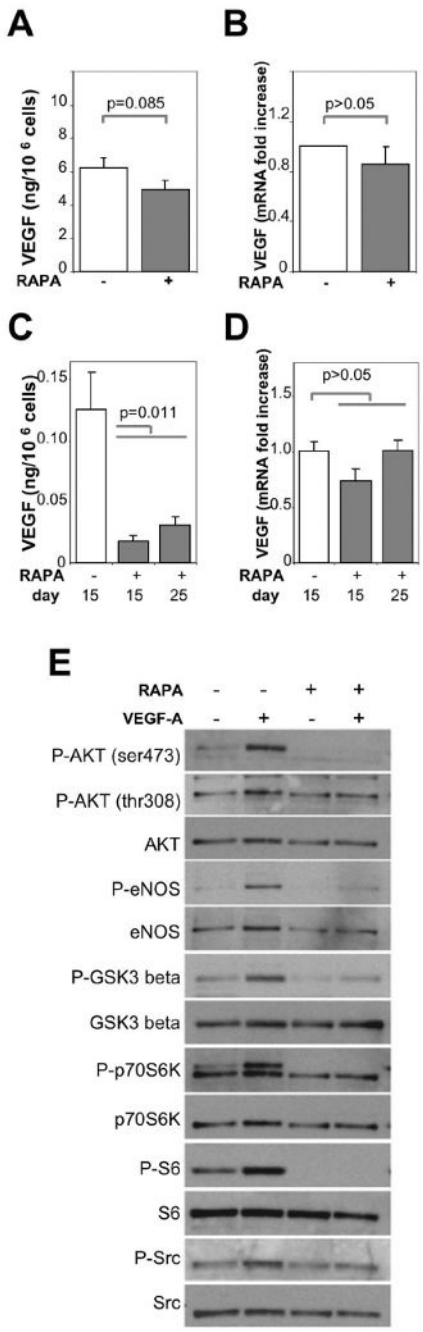

Figure 3. Effects of Rapa treatment on VEGF secretion and signaling

(A) VEGF was measured by specific ELISA in the culture supernatants of BC-1 cells incubated for 3 days in medium only (white bars) or medium supplemented with 100 nM Rapa (grey bars). The results reflect the means (±SE) of 8 experiments. **(B)** VEGF mRNA levels were measured by real time PCR in BC-1 cells incubated for 3 days in medium only (white bars) or medium supplemented with Rapa (grey bars). The results are expressed as fold increase compared to the mean of control BC-1 cells and reflect the group means (±SE). **(C)** VEGF was measured by ELISA in the ascites of control (white bars) and Rapa-treated

(grey bars) mice. The results reflect the group means (±SE). **(D)** RNA from PEL cells recovered from control (white bars) and Rapa-treated (grey bars) mice was extracted and VEGF mRNA levels were quantified by real time PCR. Results are expressed as fold increase compared to the mean values in the control (±SE). **(E)** HUVEC were incubated (0.5×10^6 cells/well, 72 h, 37°C) in maintenance medium only or with 100nM Rapa, washed and exposed (30 min, 37°C) to recombinant VEGF (100ng/ml). Cell lysates were immunoblotted with specific antibodies. The results reflect probing and stripping of a single membrane.

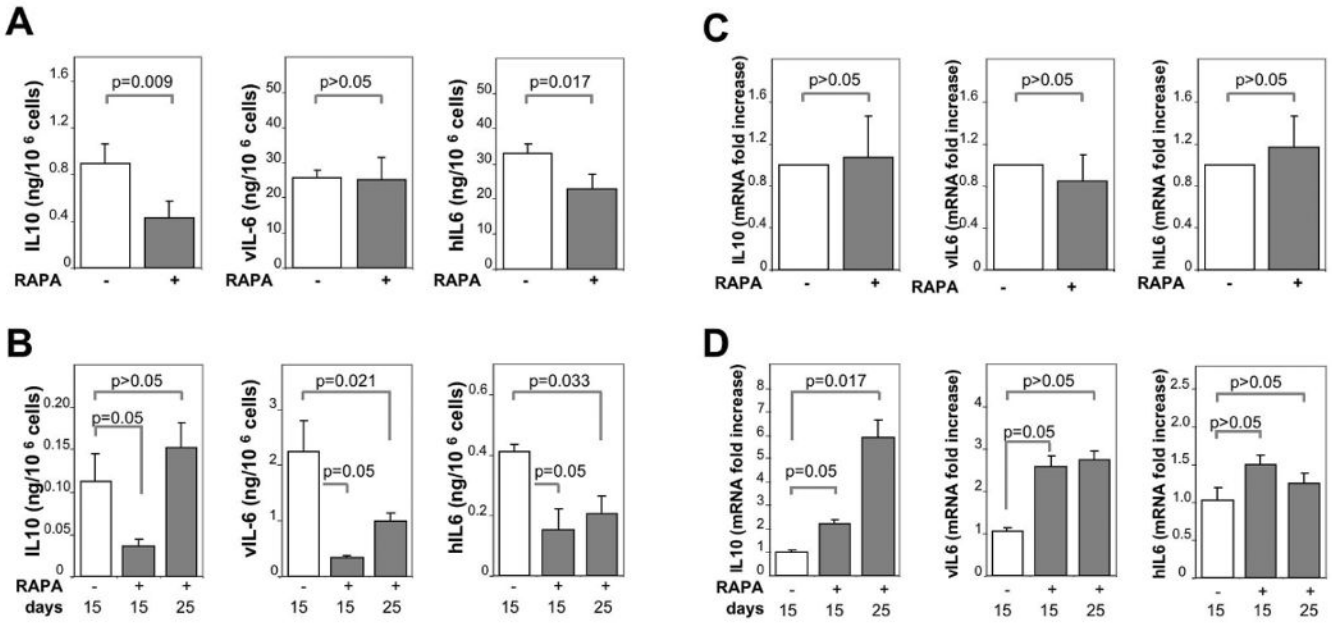

Figure 4. Effects of Rapa on autocrine growth factor expression in vitro and in vivo

(A) hIL-10, vIL-6 and hIL-6 were measured by specific ELISAs in the culture supernatants of BC-1 cells incubated for 3 days in medium only (white bars) or medium supplemented with 100nM Rapa (grey bars). The results reflect the means (±SE) of 8 samples. **(B)** hIL-10, vIL-6, hIL-6 were measured by ELISA in the ascites of control (white bars, n=3) and Rapa-treated (15, n=3 and 25, n=7 days; grey bars) mice. The results reflect the group means (±SE). **(C)** RNA was extracted from BC-1 cells incubated for 3 days in medium only (white bars) or medium supplemented with 100nM Rapa (grey bars); hIL-10, vIL-6 and hIL-6 mRNA levels were quantified by real time PCR. The results are normalized for GAPDH content and are expressed as RNA fold increase compared to untreated cells (±SE). The results reflect the means (±SE) of 6 samples. **(D)** RNA from PEL cells recovered from control (white bars, n=3) and Rapa-treated (grey bars, day 15, n=3; day 25, n=7) mice was extracted; hIL-10, vIL-6, hIL-6 mRNA levels were quantified by real time PCR. The results are expressed as fold increase compared to mean of controls and reflect the group means (±SE).

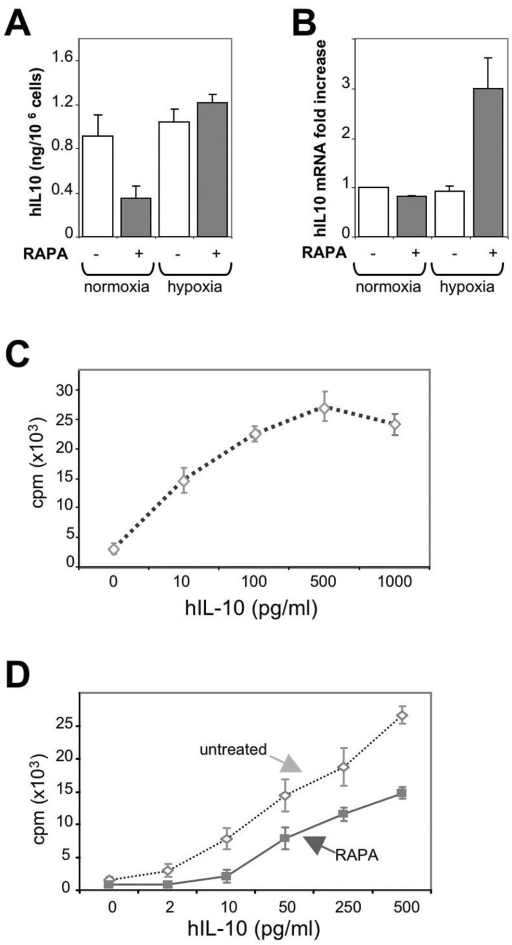

Figure 5. Effects of hypoxia on IL-10 expression and response to Rapa

BC-1 cells were incubated for 3 days in medium only or medium supplemented with 100 nM Rapa under normoxic and hypoxic conditions. **(A)** hIL-10 was measured by specific ELISA in the culture supernatants recovered at the end of incubation. White bars reflect medium only, grey bars reflect medium with Rapa. The results reflect the means (±SE) of 4 experiments. **(B)** hIL-10 mRNA levels were quantified by real time PCR in BC-1 cells recovered at the end of incubation. The results are expressed as fold increase compared to the mean of untreated control, and reflect the means (±SE) of 4 experiments. **C**) IL-10 dose-dependently promotes BC-1 cell proliferation. BC-1 cells were cultured (2×10^4 cells/ml, 37°C) in RPMI1640 medium with 2.5μg/ml transferrin, 0.25mM sodium pyruvate, 0.25 mg/ml BSA and 5 μM HEPES. **D**) BC-1 cells were cultured in medium only (described in C; diamonds) or with addition of 100nM Rapa (squares), with or without IL-10. Proliferation was measured by ^{3}H thymidine incorporation during the final 6 h of a 3-day culture.