Rapamycin inhibits primary and metastatic tumor growth by antiangiogenesis: involvement of vascular endothelial growth factor

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Conventional immunosuppressive drugs have been used effectively to prevent immunologic rejection in organ transplantation. Individuals taking these drugs are at risk, however, for the development and recurrence of cancer. In the present study we show that the new immunosuppressive drug rapamycin (RAPA) may reduce the risk of cancer development while simultaneously providing effective immunosuppression. Experimentally, RAPA inhibited metastatic tumor growth and angiogenesis in *in vivo* mouse models. In addition, normal immunosuppressive doses of RAPA effectively controlled the growth of established tumors. In contrast, the most widely recognized immunosuppressive drug, cyclosporine, promoted tumor growth. From a mechanistic perspective, RAPA showed antiangiogenic activities linked to a decrease in production of vascular endothelial growth factor (VEGF) and to a markedly inhibited response of vascular endothelial cells to stimulation by VEGF. Thus, the use of RAPA, instead of cyclosporine, may reduce the chance of recurrent or *de novo* cancer in high-risk transplant patients.

Among the most serious complications of general immunosuppressive therapy in organ transplantation is the high risk of the recurrence of neoplastic tumors and the development of *de novo* cancer. For example, recent studies in individuals with bronchioloalveolar carcinoma who received a lung transplant showed a 57% tumor recurrence rate[1]. Among individuals receiving a liver transplant for cholangiocarcinoma, 51% have tumor recurrence within 2 years[2]. With regard to *de novo* malignancies, a conservative report indicates that immunosuppressed organ allograft recipients have a 3–4-fold increased risk of developing cancer in general and a 20–500-fold higher incidence of certain types of cancer[3]. Cancer has therefore become a major cause of death in patients otherwise successfully treated by organ transplantation. One approach to addressing this problem is to identify drugs with effective immunosuppressive but low proneoplastic, or even some antineoplastic, properties. In the present study we present evidence that rapamycin (RAPA) may fill these diverse needs.

RAPA is a bacterial macrolide with antifungal and immunosuppressant activities[4,5]. It forms a complex with the FK binding protein complex (FKBP-12) that binds with high affinity to the mammalian target of rapamycin (mTOR). This interaction causes dephosphorylation and inactivation of p70S6 kinase which, when activated, stimulates the production of ribosomal components necessary for protein synthesis and cell-cycle progression[6]. This activity, which effectively blocks IL-2 stimulation of lymphocyte division, is the basis for the recent successful clinical use of RAPA to prevent allograft rejection in organ transplantation[7,8]. In comparison, the most widely recognized immunosuppressive drug, cyclosporine (CsA), binds to a different intracellular molecule, cyclophilin, ultimately leading to inhibition of T-cell proliferation by preventing IL-2 transcription[9]. Notwithstanding the mechanistic effects of these drugs on T cells, concerns about the side effects of immunosuppression on cancer development in transplant patients have brought a new scientific perspective and focus to their study. Most evidence suggests that conventional CsA immunosuppression promotes, rather than inhibits, the development of cancer[10]. Here we describe experiments indicating that immunosuppressive doses of RAPA have an entirely opposite effect, whereby the drug's potent antiangiogenic activities strongly inhibit tumor growth in mice. We also provide evidence that the antiangiogenic effect of RAPA is linked to reduced production of VEGF and to blockage of VEGF-induced endothelial cell signaling.

Metastatic tumor growth in the liver

To determine the effect of RAPA and CsA on metastatic tumor growth, we injected syngenic CT-26 adenocarcinoma cells intraportally into BALB/c mice, simulating metastasis of colon cancer to the liver. Histologic analysis of hepatic tumor-replacement area showed a marked decrease in the metastatic area in RAPA-treated mice compared to saline controls (Fig. 1*a*). Livers from RAPA-treated mice had small, avascular, disseminated metastatic foci (Fig. 1*b*). In contrast, CsA-treated mice had a high mean tumor replacement area and extensive neovascularization within the fast-growing metastases. Thus

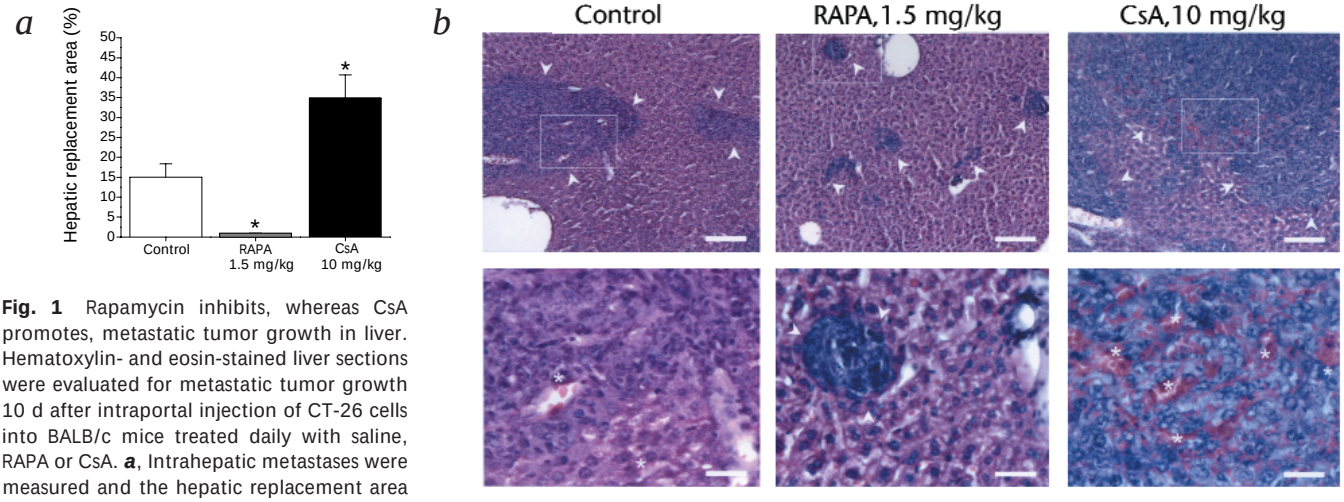

Fig. 1 Rapamycin inhibits, whereas CsA promotes, metastatic tumor growth in liver. Hematoxylin- and eosin-stained liver sections were evaluated for metastatic tumor growth 10 d after intraportal injection of CT-26 cells into BALB/c mice treated daily with saline, RAPA or CsA. ***a***, Intrahepatic metastases were measured and the hepatic replacement area determined. Each bar represents the mean ± s.e.m. from 7 mice per group. *, $P < 0.05$ versus control. ***b***, Histological sections of tumor-bearing livers on day 10 from mice treated with saline (left), RAPA (middle) or CsA (right). The upper and lower panels show representative low (bar = 200 μm) and high magnification (bar = 50 μm) views, respectively. Asterisks indicate tumor vessels and arrowheads indicate tumor borders.

in this liver metastasis model, RAPA and CsA had opposite effects on metastatic tumor growth.

Tumor growth in the dorsal skin-fold chamber

To further test how immunosuppressive drugs affect tumor development, we used intravital microscopy to examine the effect of RAPA and CsA on tumor growth and angiogenesis in dorsal skin-fold chambers. Photomicrographs representative of the time-course of tumor growth and neovascularization after the implantation of CT-26 cells into the transparent chamber of control, RAPA- and CsA-treated mice are shown in Figure 2. In the saline-treated controls, progressive tumor growth occurred over an observation period of 11 days. During the avascular phase of tumor development, tumor cell masses became visible as a shadowy area by days 3 and 5 (Fig. 2 *a-c*). Also by day 5, tumors began to induce a strong angiogenic response, as evidenced by the development of a plexus of newly formed tumor vessels that continued to expand throughout the 11-day observation period (Fig. 2*d–g*). Compared to controls, RAPA-treated mice showed less growth and neovascularization of the tumors. The inhibition of tumor growth was paralleled by a decrease in neoangiogenesis, which was visually evident from the lack of tumor vessels on days 7 and 9 (Fig. 2*d* and *e*). By day 11, vessel formation was visible, but predominantly in the central tumor area (Fig. 2*f* and *g*). The same low-magnification photomicrograph shows that, in contrast to control tumors (Fig. 2*f*), an antiangiogenic effect of RAPA was maintained in the peripheral tumor zone. This general pattern of tumor inhibition by RAPA was clearly different from the effects of CsA. CsA treatment promoted relatively early neovascularization of tumors in mice (Fig. 2*c*), which continued to progress at an accelerated rate between days 7 and 11 (Fig. 2*d–f*) as compared to control mice. Furthermore, by day 11, tumors from CsA-treated mice developed an advanced vascular network (Fig. 2*f* and *g*).

To confirm these visual observations, we carried out multiple quantitative analyses on the tumors in dorsal skin-fold chambers. As expected, RAPA-mediated inhibition of angiogenesis was identifiable on day 11, with measurements showing a smaller vascularized tumor area (Fig. 3*a*) and lower vascular density (Fig. 3*b*) in treated mice than in controls. Promotion of angiogenesis by CsA was evident from the high tumor vessel density, although the percentage of tumor area containing vessels (vascular area) was not significantly different. Corresponding measurements of tumor dimensions showed a growth dependency on angiogenesis that was reflected by a small tumor area and volume with RAPA treatment versus control (Fig. 3*c* and *d*). In contrast, the extensive angiogenesis in CsA-treated mice supported tumors with a large area and volume. Thus, results from this model indicate that immunosuppressive doses of RAPA, but not CsA, can suppress tumor growth by inhibiting angiogenesis.

Influence of rapamycin on VEGF production

Because VEGF is one of the central regulators of vessel development, we were interested in testing whether RAPA could affect angiogenesis by influencing its production *in vitro*. An enzyme-linked immunosorbent assay (ELISA) of CT-26 adenocarcinoma cell culture supernatants showed that RAPA inhibited the secretion of VEGF, whereas CsA did not have a significant effect (Fig. 4*a*). To rule out the possibility that the VEGF effect was specific to CT-26 cells, the same effect of RAPA was confirmed with B16 melanoma cells (Fig. 4*a*).

Encouraged by these *in vitro* results, we tested whether the therapeutic concentrations of RAPA used after organ transplantation could also result in reduced VEGF *in vivo*. Serum samples taken either from non-tumor-bearing mice or from mice 10 days after they had received an intraportal injection of CT-26 tumor cells showed that RAPA treatment led to a reduction in VEGF (Fig. 4*b*). Therapeutic use of CsA caused VEGF to increase slightly, possibly as a result of production from the increased tumor cell mass. For the same reason, we cannot rule out the possibility that the decreased tumor mass in RAPA-treated mice could have contributed to the lowering of serum VEGF. VEGF concentrations were lower, however, in RAPA-treated mice than in mice without tumors, indicating that endogenous VEGF production by the host was also affected.

To investigate whether RAPA inhibits angiogenesis through the downregulation of VEGF mRNA in tumor cells, or through upstream VEGF regulators such as, for example, hy-

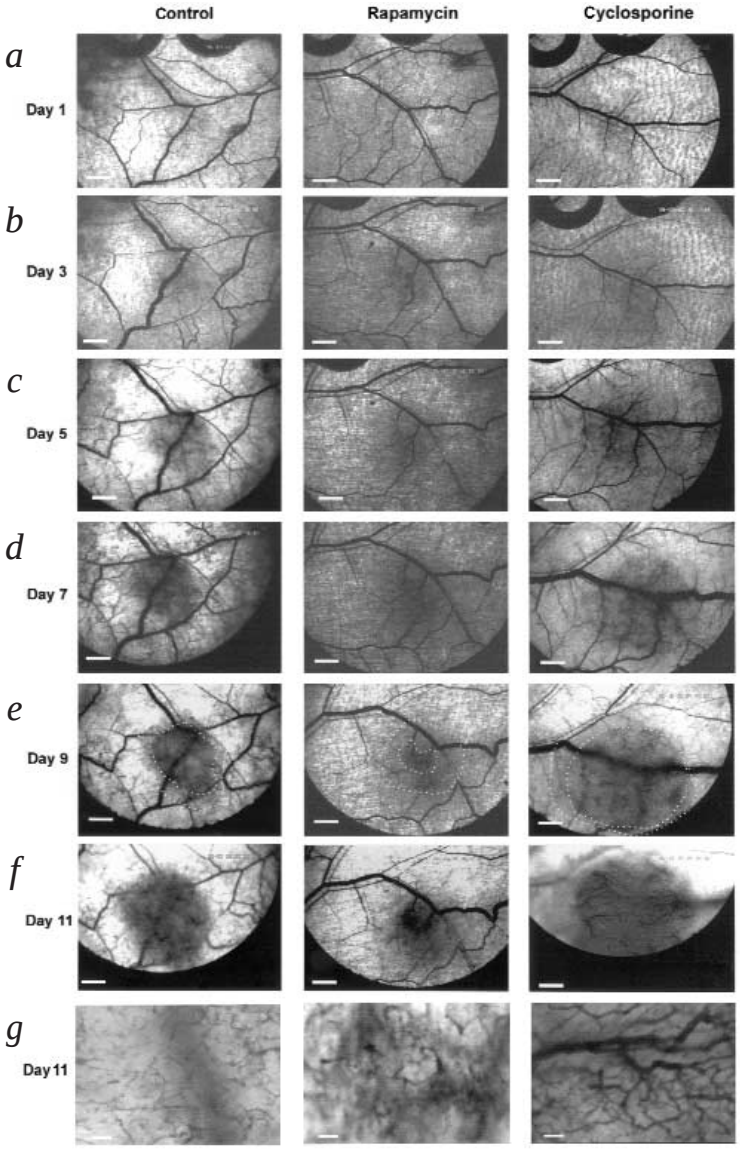

Fig. 2 Rapamycin inhibits tumor angiogenesis, whereas CsA stimulates tumor neovascularization. Angiogenesis and tumor development were monitored in dorsal skin-fold chambers after implantation of CT-26 cells in BALB/c mice. ***a–f***, Photomicrographs of representative tumors from mice treated with saline (left column), RAPA (middle column) or CsA (right column) on days 1, 3, 5, 7, 9 and 11, respectively. In the avascular stage (day 1–3), there was some initial growth of tumors (shadowy areas) for all treatments. After day 5, tumors in saline and CsA-treated mice induced a relatively large, homogenously distributed vascular plexus compared to RAPA-treated mice. Dotted lines indicate the vascularized zone in relation to the entire tumor area on day 9. On day 11, an advanced hierarchy of small vessels branching into larger draining vessels was evident in CsA-treated mice, compared to controls or RAPA-treated mice. ***g***, High-magnification views of the same tumors on day 11 show normal, interconnected, small tumor vessels in the control mouse. In contrast, tumor vessels in the RAPA-treated mouse show blunt ends, few connections and abrupt changes in diameter. Well-developed vessels can be seen in the tumor from the CsA-treated mouse. Scale bar, 1 mm (*a–f*); 0.1 mm (*g*).

poxia-inducible factor 1α (HIF-1α)[11,12] and transforming growth factor-β (TGF-β)[13–15], we cultured B16 tumor cells with and without 0.1 μg/ml RAPA for 10 h and isolated total RNA for real-time RT–PCR. Amounts of VEGF mRNA were slightly lower (4-fold) in RAPA-treated cells than in controls, but neither HIF-1α nor TGF-β mRNA seemed to be up- or down-regulated in the presence of RAPA (Fig. 4*c*). A higher dose of RAPA (1 μg/ml) did not further decrease VEGF mRNA and did not alter HIF-1α or TGF-β mRNA concentrations (data not shown). Together, these studies suggest that RAPA treatment reduces but does not decisively block VEGF production by tumor cells.

Effect of RAPA on proliferation and VEGF-mediated angiogenesis

We next explored whether the antitumor activity of RAPA could be related to an antiproliferative effect acting either directly on tumor cells or on vascular endothelial cells. Bromodeoxyuracil (BrdU) incorporation experiments showed that CT-26 and B16 tumor cell proliferation decreased moderately in the presence of RAPA, but only at the highest concentration tested (1 μg/ml, Fig. 5*a*). Human umbilical-cord vein endothelial cells (HUVECs) were very sensitive to RAPA, with a significant effect at 0.01 μg/ml—again raising the possibility that VEGF may be involved in the RAPA effect. Our hypothesis, however, was based on the assumption that adequate VEGF was present but VEGF stimulation of endothelial cells was inhibited. To test this theory, HUVECs cultured under minimal serum-deprived conditions, were stimulated with recombinant VEGF with or without RAPA. Results showed that RAPA markedly reduced VEGF-induced HUVEC proliferation in a dose-dependent manner (Fig. 5*a*). We therefore examined the effects of RAPA on VEGF stimulation of HUVEC tubular formation morphogenesis and found that VEGF-induced HUVEC tubular formation was completely abrogated by concentrations of RAPA as low as 0.01 μg/ml (Fig. 5*b*). Thus, VEGF-dependent HUVEC proliferation and morphogenesis seem to be very sensitive to the effects of RAPA.

Treatment of tumors with rapamycin

We next tested the effects of RAPA on the growth of established subcutaneous CT-26 tumors (Fig. 6*a*). The tumors rapidly grew in untreated control mice, resulting in the death of all mice by 2 weeks. By contrast, mice receiving relatively low doses of RAPA (1.5 mg/kg/d) showed a slightly delayed increase in tumor size during the first 20 days. At this time point the tumors began to regress markedly and the animals had a 100% survival rate. Later, widespread tumor necrosis occurred (Fig. 6*d*). The lowest dose of RAPA (0.15 mg/kg/d) delayed tumor growth slightly, but the mice died by day 23 and the tumors never entered a regression phase. Notably, a high dose of RAPA (15 mg/kg/d) caused a more pronounced delay in tumor development during the first 3 weeks, but after this the tumors began to grow again rapidly and the mice died shortly thereafter. An even higher dose (30 mg/kg/d) resulted in the death of all mice by day 17 (data not shown). The antitumor effect of RAPA was not specific to CT-26 tumors or to BALB/c mice, as a similar treatment protocol for subcutaneous B16 tumors in C57BL/6J mice also caused a marked decrease in tumor vol-

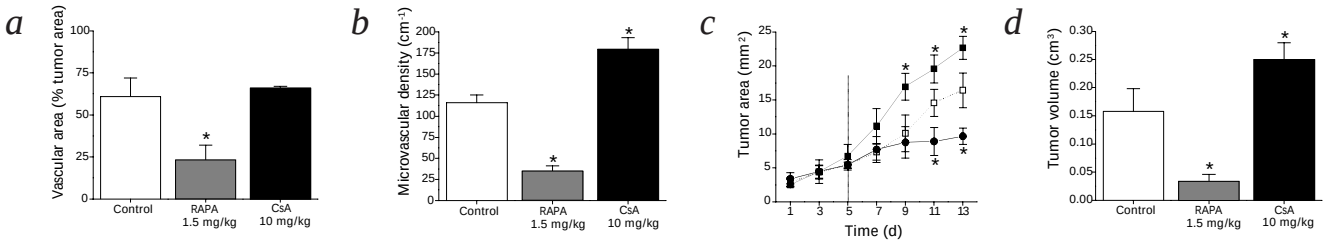

Fig. 3 CT-26 tumors in transparent chambers are markedly smaller and have less vascularization with rapamycin compared to CsA treatment. Tumor size and vascularization were determined after inoculation of CT-26 cells into the transparent chambers of control and drug-treated mice. ***a***, Effect of RAPA or CsA on vascular area on day 11. ***b***, Effect of RAPA or CsA on microvascular density on day 11. ***c***, Tumor area in mice treated until day 13 with saline (□), RAPA (●) and CsA (■). The vertical line indicates the normal transition from prevascular angiogenesis-independent to angiogenesis-dependent growth. ***d***, On day 13, tumors were excised and the volume determined. All results are the mean ± s.e.m. of 7 mice per group. *, $P < 0.05$ versus saline control.

ume after a 3–4-week period (Fig. 6*b*). Here also, the dominant feature of the B16 tumors after 4 weeks was massive tumor necrosis (Fig. 6*e*). These data indicate that RAPA significantly inhibits the growth of established vascularized tumors, and this effect is best realized with relatively low (normal immunosuppressive) doses of drug.

To test whether orthotopically placed tumors respond in a similar way to the effects of RAPA, we injected CT-26 tumor cells into the cecal wall of BALB/c mice and treated the mice with RAPA. Excised tumors from RAPA-treated mice on day 12 were 87% smaller than those from control mice (Fig. 6*c* and *f*). In addition, immunohistologic staining of tumor endothelial cells in RAPA-treated mice showed a marked decrease in the number of blood vessels (Fig. 6*g*). Together, these data indicate that the antiangiogenic–antitumor effect of RAPA occurs with orthotopically grown tumors and that this effect is not dependent on a subcutaneous tumor location.

Discussion

Our results indicate that RAPA and CsA have contrasting effects on tumor development that could be crucial in addressing the problem of cancer in immunosuppressed patients. In our tumor cell metastasis studies, we found that doses of RAPA roughly equivalent to those used in organ transplantation strongly inhibited tumor growth in the mouse liver. In contrast to the abnormally large, vascularized tumor masses seen with CsA treatment, metastatic foci in the liver of RAPA-treated mice had few blood vessels and were too small to require angiogenesis[16,17]. We considered two basic theories to explain the potential antitumor effects of RAPA. First and most simply, RAPA might directly inhibit the proliferation of tumor cells. Our results did not strongly support this, however, as RAPA inhibited tumor proliferation only slightly at the highest concentration tested (1 μg/ml) and had no apparent effect in a more relevant therapeutic range (<1.0 μg/ml). The blood concentrations of the drug in mice receiving 1.5 mg/kg/d were approximately 0.04 μg/ml at the trough and approximately 0.8 μg/ml at 2 hours after administration (data not shown). RAPA has antiproliferative activity against normal nonimmunologic cells[6] and colon-38 tumor cells[18], but only at extremely high doses of drug (100–400 mg/kg/d intraperitoneally), consistent with our data. Therefore, the inhibitory effect of normal immunosuppressive doses of RAPA on tumor growth in our model is probably not due to an antiproliferative effect on tumor cells.

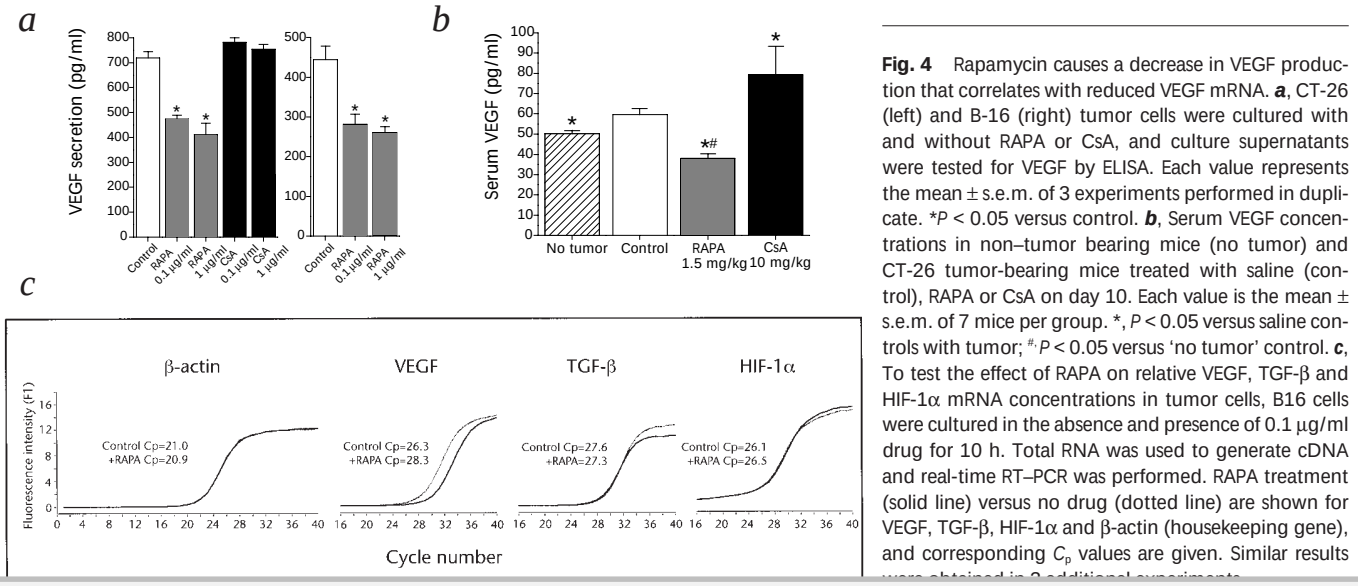

Fig. 4 Rapamycin causes a decrease in VEGF production that correlates with reduced VEGF mRNA. ***a***, CT-26 (left) and B-16 (right) tumor cells were cultured with and without RAPA or CsA, and culture supernatants were tested for VEGF by ELISA. Each value represents the mean ± s.e.m. of 3 experiments performed in duplicate. *$P < 0.05$ versus control. ***b***, Serum VEGF concentrations in non–tumor bearing mice (no tumor) and CT-26 tumor-bearing mice treated with saline (control), RAPA or CsA on day 10. Each value is the mean ± s.e.m. of 7 mice per group. *, $P < 0.05$ versus saline controls with tumor; #, $P < 0.05$ versus 'no tumor' control. ***c***, To test the effect of RAPA on relative VEGF, TGF-β and HIF-1α mRNA concentrations in tumor cells, B16 cells were cultured in the absence and presence of 0.1 μg/ml drug for 10 h. Total RNA was used to generate cDNA and real-time RT–PCR was performed. RAPA treatment (solid line) versus no drug (dotted line) are shown for VEGF, TGF-β, HIF-1α and β-actin (housekeeping gene), and corresponding C_p values are given. Similar results

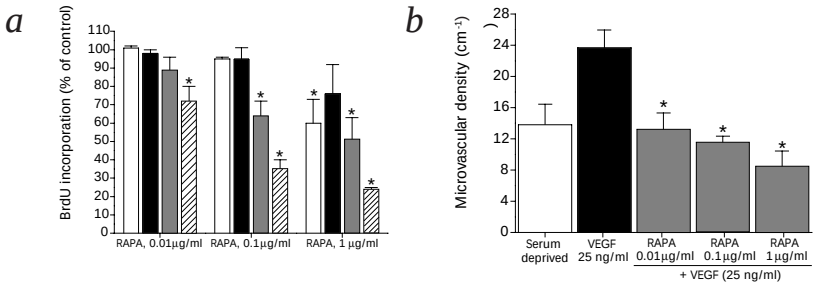

Fig. 5 Rapamycin inhibits tumor cell proliferation and VEGF-dependent HUVEC proliferation and tubular formation. ***a***, The effect of RAPA on the proliferation of CT-26 (□) and B16 (■) tumor cells and HUVEC cells (■) determined by BrdU incorporation (shown as % of control BrdU incorporation). An additional experiment was performed with HUVECs whereby serum-deprived cells were stimulated with recombinant VEGF (▨). BrdU incorporation was measured and is expressed as the percent of control (VEGF-stimulated cultures). All data represent the mean ± s.e.m. from 3 experiments. *, $P < 0.05$ versus control. Compared to serum deprivation, optimal culture supplementation increased BrdU incorporation by 79%, and recombinant VEGF in serum-deprived medium increased incorporation by 61%. ***b***, The effect of RAPA on VEGF-induced HUVEC tubular formation as tested by culturing HUVECs in serum-deprived medium only or in serum-deprived medium containing recombinant VEGF ± RAPA; vascular tube density was measured 8 h later. Results represent the mean ± s.e.m. of 3 experiments. *, $P < 0.05$ versus VEGF with no drug.

Our second theory about the RAPA antitumor effect was based on our early observation that small liver metastases in RAPA-treated mice had few blood vessels. From this, we predicted that RAPA could mediate its effect through inhibition of tumor vascularization. Using a dorsal skin-fold chamber model, which is well established for the specific study of tumor neoangiogenesis[16,17,19], we found that *in vivo* treatment with RAPA strongly inhibits tumor angiogenesis and associated tumor growth. Also consistent with the liver metastasis experiments, CsA treatment caused a completely opposite effect, where relatively large tumors formed and were supported by advanced neovascularization. One consideration in the interpretation of data from the dorsal skin-fold chambers is that tumors developed in an ectopic location. To elucidate possible host microenvironmental influences[20,21], it would be useful to carry out angiogenesis-specific studies of tumors in an orthotopic location. In regard to this question, we note that CD31 staining of vascular endothelial cells in orthotopically placed CT-26 tumors showed a significant reduction of tumor vessels with RAPA treatment. Data presented here provide the first clear evidence that normal immunosuppressive concentrations of RAPA and CsA have divergent effects on angiogenesis and that RAPA has potent tumor inhibiting antiangiogenic effects.

From a mechanistic perspective, our study suggests the antiangiogenic effect of RAPA is related to VEGF antagonism at two separate levels. At one level, VEGF production is reduced. This is evidenced by *in vitro* experiments showing that RAPA diminishes VEGF secretion by tumor cells, and is further corroborated by the reduced VEGF mRNA concentrations measured in the same cells. This general observation was also evident *in vivo*, where serum VEGF was lower in mice treated with RAPA. Although we speculated that the decrease in VEGF production could be related to the reported upstream regulatory effects of either HIF-1α[11,12] or possibly TGF-β[13–15], we found no evidence for quantitative changes in these molecules at the mRNA level in the presence of RAPA. Our results do not, however, rule out the possibility that RAPA disturbs angiogenesis-dependent intracellular pathways downstream of these molecules, or that the mRNA translation or protein degradation rates of HIF-1α or TGF-β are modified by RAPA. These arguments notwithstanding, we suspected that the rather modest decrease in VEGF production by tumor cells could not fully account for the potent antiangiogenic effect. In addition, because it can be argued that many tumor cells produce excessive amounts of proangiogenic factors, including VEGF[16,22], a moderate reduction in VEGF production may not be sufficient to impede neoangiogenesis fully. Therefore, assuming adequate amounts of VEGF, we tested whether RAPA could be inhibiting angiogenesis at a second level, where receptor-mediated stimulation of vascular endothelial cells occurs. This hypothesis was based on evidence that the PI3K-p70S6 kinase intracellular signaling pathway is required for VEGF stimulation of endothelial cells[23,24]. In this regard, one study[25] has shown that RAPA inhibition of p70S6 kinase blocks PMA-induced tube formation in three-dimensional HUVEC cultures. VEGF receptor-2 signaling in pig endothelium has also been linked to the activation of the PI3K-p70S6 pathway[26]. Indeed, our data indicating that RAPA markedly inhibited VEGF-dependent HUVEC proliferation and completely abrogated VEGF-induced tubular formation favors this mechanistic explanation. RAPA concentrations that caused these *in vitro* effects on endothelial cells were well within the range measured in serum after RAPA treatment for tumors and also are similar to those used in organ transplantation to prevent allograft rejection.

The convincing antiangiogenic effects of RAPA in our earlier studies led us to test if this drug had antitumor effects in a more clinically relevant situation. The potency of RAPA in this respect was revealed by its ability to control and effectively shrink established subcutaneous and orthotopic tumors in mice. The hypothesis that RAPA affects tumor growth primarily through antiangiogenesis is supported by our data showing fewer CD31-positive blood vessels in orthotopic tumors in mice treated with RAPA compared to untreated controls. The theory is further supported by our observation of an atypical dose-response effect on established tumors. A relatively low, non-cytotoxic dose of RAPA caused the tumors to regress only after they became increasingly dependent on angiogenesis. This observation is consistent with the low-dose, delayed effects on tumors typically seen with other reported antiangiogenic treatment protocols[27–30]. The greater delay in the early development of established tumors subjected to high doses of RAPA may be explained by a modest antiproliferative effect, as has been reported with very high *in vivo* doses of RAPA[18]. We speculate that antiproliferative effects associated with these high RAPA concentrations may slow early tumor growth but also weaken the general condition and immunity of the mouse to a point where tumor growth has the advantage. Together, these experiments suggest that immunosuppressive doses of RAPA may have long-term antiangiogenic effects that control tumor growth

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