Loss of Tsc1 or Tsc2 Induces Vascular Endothelial Growth Factor Production through Mammalian Target of Rapamycin

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Abstract

Mutation in either TSC1 or TSC2 causes the autosomal dominant disorder tuberous sclerosis, in which widespread hamartomas are seen, some of which have a high level of vascularization. Tuberous sclerosis complex (TSC) gene products negatively regulate mammalian target of rapamycin (mTOR) activity. We found that vascular endothelial growth factor (VEGF) is secreted by Tsc1- or Tsc2-null fibroblasts at high levels compared with wild-type cells. In Tsc1+/− mice, serum levels of VEGF were increased and appeared to be associated with the extent of tumor development. Rapamycin, an mTOR inhibitor, reduced the production of VEGF by Tsc1- and Tsc2-null fibroblasts to normal levels. Moreover, short-term treatment of Tsc1+/− mice with rapamycin at 20 mg/kg led to some changes in tumor morphology and a reduction in serum VEGF levels. These observations have three implications. First, TSC gene products regulate VEGF production through a mTOR signaling pathway. Second, serum VEGF levels may be a useful clinical biomarker to monitor the progression of TSC-associated lesions. Last, rapamycin or related inhibitors of mTOR may have therapeutic benefit in TSC both by direct tumor cell killing and by inhibiting the development of TSC lesions through impairment of VEGF production.

Introduction

TSC3 is an autosomal dominant genetic disorder characterized by multigland hamartomatosis as a result of mutations in either TSC1 or TSC2 (1). TSC1 and TSC2 form a protein complex (2, 3), and mutations in either gene lead to similar clinical phenotypes. TSC follows the two-hit model of tumor suppressor genes in that TSC lesions often show loss of heterozygosity for the wild-type allele (4, 5).

Some TSC hamartomas, including renal AMLs and skin angiomyfibroma, are characterized by numerous abnormal vascular channels (1). AMLs are typically highly vascular, progress requiring clinical intervention in about 10% of TSC patients, and are associated with both renal failure and sudden death due to massive hemorrhage (1). We have described Tsc mouse models that have been developed by gene targeting (6, 7). Both Tsc1+/− and Tsc2+/− mice develop liver hemangiomas, renal cystadenomas, and, in rare cases, angiosarcomas. The liver hemangiomas are characterized by extensive abnormal vasculogenesis (Fig. 3, A and B), and fatal hemorrhage from these lesions is a common event (7). Thus, a high level of angiogenesis occurs in both human and mouse Tsc lesions.

Angiogenesis is required for tumor growth and metastasis and is triggered by both oncogene activation and tissue hypoxia (8, 9). VEGF is the major angiogenic growth factor generated by malignant cells and induces angiogenesis and vasculogenesis in vivo through direct interaction with the tyrosine kinase receptors VEGF receptor-1 (Flt) and VEGF receptor-2 (Flk-1/KDR) on endothelial cells (8, 9).

Recent studies have elucidated an important role for the TSC gene products in a conserved signaling pathway that includes mTOR (7, 10–12). Both Tsc1-null and Tsc2-null cell lines show an increase in the phosphorylation of S6 kinase and 4E-BP1 that is inhibited by rapamycin, consistent with constitutive activation of mTOR (7, 10). Here we investigated the hypothesis that loss of Tsc1 or Tsc2 leads to secretion of VEGF, which is also dependent on mTOR signaling, and that this event contributes to the vascular nature of Tsc lesions.

Materials and Methods

Tsc1-null and Tsc2-null fibroblast cell lines prepared from E10.5 embryos were prepared as described previously (7). They were cultured in DMEM with 10% FCS at 5% CO2, in parallel with littermate-derived control fibroblast cell lines. A cell proliferation (MTT) assay measuring NADPH and NADH production was performed after plating 4 × 104 fibroblasts in 6-well plates. Six hours after plating, fresh DMEM with 1% FCS alone or supplemented with 10 nm rapamycin was added, and cells were incubated for 48 h. Media were collected at 24 or 48 h, and VEGF measurements were determined by ELISA for mouse VEGF (Oncogene Research Products). This ELISA method was also used to assess serum VEGF levels in Tsc1+/− and wild-type mice, both in strain 129/Sv.

Rapamycin was administered to mice by i.p. injection at 20 mg/kg daily for 4 days. It was reconstituted in absolute ethanol at 10 mg/ml and diluted in 5% Tween 80 and 5% PEG-400 before injection.

Necropsy and pathological analysis was performed by careful inspection of the external surface of both kidneys and the liver for involvement by cystadenomas and hemangioma, respectively. The kidneys were sliced at 1 mm in entirety for pathological review. For immunohistochemistry, 5-μm paraffin-embedded tissue sections were deparaffinized in xylene and rehydrated in an ethanol/water series. Sections were stained by the peroxidase method (ImmunoCruz kit; Santa Cruz Biotechnology) using primary antibodies against VEGF (Santa Cruz Biotechnology), and pS6 Ser 235/236 antibodies (Cell Signaling).

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3 The abbreviations used are: TSC, tuberous sclerosis complex; mTOR, mammalian target of rapamycin; VEGF, vascular endothelial growth factor; AML, angiomyolipoma; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; HIF-1α, hypoxia-inducible factor-1α.
**Results and Discussion**

Strong VEGF expression was seen by immunoblot (Fig. 1A) and immunohistochemistry (Fig. 3, C and E) analyses of mouse liver hemangiomas from Tsc1\(^{-/-}\) and Tsc2\(^{-/-}\) mice, in comparison with normal liver (Figs. 1A and 3D). These immunoblot results underestimate the extent of VEGF production by these tumors because they are infiltrative and impossible to dissect cleanly from adjacent liver. Unrecognized hemorrhage involvement may explain the small amount of VEGF detected in “normal” liver from a Tsc1\(^{-/-}\) mouse (Fig. 1A, Lane 1); VEGF was not seen in two other Tsc1\(^{-/-}\) mouse liver samples (data not shown). Similarly, VEGF expression was high in Tsc2\(^{-/-}\) and Tsc2\(^{-/-}\) renal cystadenomas in comparison with normal kidney tissue (Figs. 1B and 3K).

Although neovascularization has been observed in brain, skin, and kidney lesions from TSC patients (13), the mechanism of this phenomenon has been uncertain, as well as whether Tsc1- or Tsc2-null cells in these tumors secrete VEGF or induce VEGF production by surrounding tissues. Therefore, we examined VEGF production by fibroblast cell lines that were null for either Tsc1 or Tsc2 (6, 7). Conditioned media from Tsc1\(^{-/-}\), Tsc2\(^{-/-}\), and wild-type fibroblast cultures were collected 24 and 48 h after plating and subjected to ELISA to measure VEGF. VEGF levels were significantly higher in the Tsc1\(^{-/-}\) and Tsc2\(^{-/-}\) cell media in comparison with media from wild-type cells (Fig. 2, A and B). Production of VEGF was significantly reduced in Tsc2\(^{-/-}\) cells transfected to express human TSC2 (Figs. 1C and 2C). In addition, Tsc2\(^{-/-}\) cells transfected with three different disease-associated (4) mutant TSC2 cDNAs continued to show high-level secretion of VEGF (Figs. 1C and 2C). These data indicate that increased production of VEGF was a direct result of loss of the Tsc2 gene product in these cells.

We then measured serum VEGF levels in Tsc1\(^{-/-}\) and wild-type mice (Table 1A). Serum VEGF levels in littermate control wild-type mice were between 50 and 91 pg/ml, whereas levels in Tsc1\(^{-/-}\) mice of similar age were between 99 and 251 pg/ml (P = 0.02, t test). Necropsy analysis showed that Tsc1\(^{-/-}\) mice with high serum levels of VEGF had significant tumor involvement of liver and/or kidney. Although the numbers studied do not permit statistical significance, the extent of liver involvement by hemangiomas appeared to be associated with the degree of serum VEGF elevation (Table 1A). VEGF levels also tended to be higher in female compared with male Tsc1\(^{-/-}\) mice, consistent with their higher rate and severity of liver hemangioma development (7).

Recently, we have shown that deficiency in either Tsc1 or Tsc2 in cultured cells results in unregulated activation of mTOR, which leads to aberrant high-level phosphorylation of S6 kinase and 4EBP1 (Ref. 7; Fig. 1C). This mTOR activation signature is sensitive to rapamycin treatment, a highly specific inhibitor of mTOR (7, 10). We reasoned that VEGF production was likely downstream of mTOR, and therefore we investigated the effect of the mTOR inhibitor rapamycin on VEGF production in Tsc-null cells. Rapamycin inhibited VEGF production by both Tsc1\(^{-/-}\) and Tsc2\(^{-/-}\) cells in a dose-dependent manner (Fig. 2, A, B, and D).

We next explored the effects of rapamycin treatment on VEGF production in vivo. Serum VEGF levels were measured in three Tsc1\(^{-/-}\) female mice (age, 10 months), before and after treatment with 20 mg/kg rapamycin i.p. daily for 4 days. Serum levels of VEGF were reduced after rapamycin in all three mice, with large reductions in two mice that began with very high VEGF levels (Table 1B). These results indicating the potent effects of rapamycin treatment on VEGF production in vitro and in vivo suggest that VEGF production is due to the constitutive activation of mTOR in Tsc1- or Tsc2-null cell lines and tumors. The reduction of VEGF levels in vivo could also have been due to tumor cell death induced by rapamycin in the Tsc1\(^{-/-}\) mice.

We therefore examined the effects of rapamycin treatment on the tumors seen in these mice. Although clear morphological changes were not apparent in liver hemangiomas sections after short-term rapamycin treatment, VEGF expression was reduced in hemangioma sections in the treated mice (Fig. 3, F and L). Kidney sections from rapamycin-treated mice showed a flattening of the epithelial cells lining the cystadenomas, with hematoxylin-positive necrotic debris in the lumen of the cysts (compare Fig. 3G, an untreated Tsc1\(^{-/-}\) mouse kidney cystadenoma, with Fig. 3H, a rapamycin-treated kidney cystadenoma). A solid adenoma from a rapamycin-treated mouse showed less dramatic cellular changes but also showed regions of apoptotic cells that were TUNEL positive (Fig. 3, I and J). These findings were not seen in control untreated mice. In addition, VEGF expression was reduced in kidney lesions from the rapamycin-treated mice in comparison with untreated mice (Fig. 3, K and L). Thus, these observations suggest that short-term treatment with rapamycin had some tumor cell kill effect. However, liver hemangiomas did not change in appearance, suggesting that rapamycin therapy led to a reduction in VEGF production before tumor cell death.

We next examined whether the inhibition of VEGF production by rapamycin was due to an antiproliferative effect on Tsc1\(^{-/-}\) or Tsc2\(^{-/-}\) cells. Using the MTT cell proliferation assay, we observed that rapamycin treatment inhibited VEGF production during a 24-h treatment interval but had no effect on Tsc2\(^{-/-}\) cell proliferation (Fig. 2, E and F). Longer-term treatment with rapamycin may have had a greater effect on growth in this assay, but the observations indicate that the effect of rapamycin on VEGF is rapid and occurs before an antiproliferative effect.

Intense investigation has led to the recognition that several different factors regulate and induce VEGF production, including...
cytokines, hormones, and hypoxia (8, 9, 14–16). Hypoxia is one of the major stimulators of VEGF gene expression in neoplastic cells and is thought to act primarily through regulation of the stability of HIF-1α (8, 9, 14, 15). However, several oncogenes, such as v-src, are known to promote VEGF production in the absence of hypoxia by increasing HIF-1α expression (17). Recent studies have demonstrated that activation of mTOR up-regulates HIF-1α expression, which results in an increase in VEGF production (18, 19). These previous results, combined with our data, suggest a model in which cells lacking either tuberin or hamartin activate mTOR, which in turn leads to increased expression of HIF-1α, resulting in stimulation of VEGF production. Rapamycin treatment, a mTOR inhibitor, reverses this effect.

In summary, our data indicate that a lack of either the TSC1 or the TSC2 gene products leads to VEGF production by both cultured cells and tumors in vivo. VEGF levels are rapidly reversed in vitro by treatment with rapamycin, before an antiproliferative effect, suggesting that increased VEGF production occurs secondary to activation of mTOR. In vivo, short-term rapamycin treatment of Tsc1−/− mice rapidly reduces VEGF levels before clear evidence of tumor cell death in liver hemangiomas, consistent with an inhibitory effect on VEGF production in Tsc1-null tumor cells.
Table 1. Serum levels of VEGF in Tsc1<sup>−/−</sup> mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Sex</th>
<th>Age (mo)</th>
<th>Liver tumor</th>
<th>Kidney tumor</th>
<th>VEGF level</th>
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<tr>
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<td>Female</td>
<td>13</td>
<td>Massive multilobar</td>
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<td>249</td>
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<tr>
<td>Tsc1&lt;sup&gt;−/−&lt;/sup&gt;</td>
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<td>Massive multilobar</td>
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<td>244</td>
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<td>9</td>
<td>nd&lt;sup&gt;b&lt;/sup&gt;</td>
<td>nd</td>
<td>154</td>
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<tr>
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<tr>
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<td>13</td>
<td>Multilobar</td>
<td>11 (&lt;1 to &gt;2 mm)</td>
<td>138</td>
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<tr>
<td>Tsc1&lt;sup&gt;−/−&lt;/sup&gt;</td>
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<td>12</td>
<td>Massive multilobar</td>
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<td>152</td>
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<td>10</td>
<td>nd</td>
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</table>

A. Serum levels of VEGF (pg/ml) and pathological findings in Tsc1<sup>−/−</sup> and wild-type control mice

B. Serum levels of VEGF in Tsc1<sup>−/−</sup> mice before and after rapamycin treatment

<sup>a</sup> Both liver and kidney tumors were assessed by gross pathological inspection.

<sup>b</sup> nd, not done, necropsy analysis not performed.

Fig. 3. Pathology of Tsc1<sup>−/−</sup> mice and effects of treatment with rapamycin. A and B, liver hemangiomia from an untreated mouse showing proliferating smooth muscle cells and abnormal vascular channels of variable size. C–F, VEGF immunohistochemistry of mouse liver shows strong expression of VEGF in untreated hemangioma (C, arrow indicates spindle-shaped cells expressing VEGF), no reactivity in normal liver from a wild-type mouse (D), no reactivity in a parallel section (to C) of a hemangioma when the primary antibody was omitted (E), and reduced VEGF expression in a hemangioma from a Tsc1<sup>−/−</sup> mice treated with rapamycin for 4 days (F). G–I, renal cystadenomas of Tsc1<sup>−/−</sup> mice. G, untreated mouse tumor shows papillary projections with elongated epithelial lining cells. H, rapamycin-treated mouse tumor shows flattened epithelial cells, with fewer papillary projections, and necrotic debris in the cyst lumen. I, rapamycin-treated mouse solid adenoma shows a more modest change in cell morphology but with regions of cellular debris. J, TUNEL stain highlights regions of apoptosis in the rapamycin-treated solid adenoma. K and L, VEGF expression in control (K) and rapamycin-treated (L) kidney tumors. Note reduced expression by the treated tumor.
These data provide further support for the concept that rapamycin may have unique therapeutic benefit for the lesions that occur in TSC patients (7, 20), potentially through both tumor cell death and inhibition of VEGF production. Our observations also suggest that serum VEGF levels could be a clinically useful biomarker to monitor the development and progression of TSC-associated lesions, particularly renal AMLs.

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References
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