Mammalian Target of Rapamycin Is the Key Effector of Phosphatidylinositol-3-OH–Initiated Proliferative Signals in the Thyroid Follicular Epithelium

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Abstract

Activation of the phosphatidylinositol-3-OH kinase (PI3K) signaling cascade is becoming increasingly recognized as a common feature of thyroid follicular neoplasms. We have recently shown that conditional loss of Pten in the mouse thyroid follicular cells is sufficient to stimulate continuous autonomous growth, leading to a homogeneously hyperplastic gland and to the development of follicular adenomas. Because the PI3K/AKT cascade can activate a plethora of different signaling pathways, it is still unclear which of these may represent the key mitogenic output of PI3K-initiated signaling. Here, we show that the in vivo proliferative response to chronic PI3K activation profoundly relies on the activation of the mammalian target of rapamycin (mTOR)/S6K1 axis, and that mTOR inhibition in Pten mutant mice and cells restores virtually normal proliferation rates, despite the presence of still elevated Akt activity, at least in part by down-regulating cyclins D1 and D3, and without affecting cell survival. [Cancer Res 2008;68(2):444–9]

Introduction

The thyroid gland is characterized, under nonpathologic conditions, by a very low proliferation index, with an estimated turnover time for the follicular cells of 8.5 years (1). However, even subtle alterations of the mechanisms governing its homeostasis may lead to increased growth and to a variety of pathologic conditions, ranging from nodular hyperplasia to neoplastic transformation. In fact, clinically silent thyroid nodules are estimated to affect up to 67% of the U.S. population (2). The view that thyroid growth is primarily induced by the pituitary-derived thyroid-stimulating hormone (TSH; ref 3) has been recently integrated by the notion that the insulin-like growth factor I/phosphatidylinositol-3-OH kinase (PI3K) axis plays an equally essential role for thyrocyte proliferation (4). In fact, the PI3K pathway has been shown, in thyroid cell culture systems, to transmit the growth factor–dependent proliferative signals (5, 6). The immediate downstream effectors of PI3K signaling are the AKT serine/threonine kinase, which regulates key processes such as proliferation, survival, cell size, and mRNA translation (7), and the tumor suppressor PTEN, which counteracts PI3K activity by dephosphorylating its product, the second messenger phosphatidylinositol 3,4,5-triphosphate (8).

Constitutive activation of the PI3K signaling cascade is now being increasingly recognized as one of the most common molecular events in thyroid follicular adenoma and carcinoma (9, 10). In addition, heterozygous mutations of the PTEN tumor suppressor, a negative regulator of this pathway, cause Cowden disease, a dominant genetic syndrome whose characteristics include multinodular goiter, adenoma, and a 10% lifetime risk for developing thyroid follicular cancer (11).

The wide variety of growth-promoting pathways controlled by the PI3K/AKT/PTEN cascade leads to the essential issue of their relative relevance and specific roles. Among these downstream effectors, activation of the mammalian target of rapamycin (mTOR) kinase has recently taken center stage, thanks to the increasing evidence that, in different cell types, it is directly responsible for the increased proliferation associated with PI3K activation (12–17). This notion has led to the development of novel therapeutic strategies based on the direct inhibition of mTOR via rapamycin and its derivatives (16).

We have recently described a genetically engineered mouse strain in which the Pten gene is selectively deleted in the thyroid follicular cells, thus constitutively activating the PI3K/Akt pathway and reproducing the molecular events taking place in a large number of thyroid follicular adenomas and carcinomas (18). Through this approach we have shown in vivo that constitutive activation of the PI3K/Akt cascade conveys a major proliferative signal in thyroid follicular cells that is sufficient to induce thyroid hyperplasia in young mice and to create fertile ground for adenoma development in older mutants. Here, we have addressed in vivo the function of the mTOR kinase as a downstream player in the mitogenic signals initiated by PI3K in the thyroid follicular cells.

Materials and Methods

Animals and treatments. PtenL/L;TPO-Cre mice (129S6/SvEv background) were bred in the Fox Chase Cancer Center (FCCC) Laboratory Animal Facility. RAD001 (everolimus; kindly provided by Dr. Heidi Lane, Novartis Institutes for Biomedical Research, Basel, Switzerland) was dissolved in water and given daily by p.o. gavage at a dose of 10 mg/kg body weight for 2 weeks, starting at age 4 weeks. At the end of the experiments, mice were euthanized and weighed. The thyroid was dissected and weighed; one lobe was fixed for pathologic analysis and the other frozen in liquid nitrogen for protein extraction.

Cells and treatments. FTC-133 cells (19) were grown in DMEM with 10% fetal bovine serum (FBS). Primary thyrocytes were isolated essentially as described (13). Briefly, thyroids were dissected from 8-week-old mice and collected in Ham’s F12/10% FBS with 100 units/mL type I collagenase (Sigma) and 1 unit/mL dispase (Roche). Enzymatic digestion was carried out for 90 min at 37°C. After digestion, isolated follicles were seeded in Ham’s F12 containing 40% Nu-Serum IV (Collaborative Biomedical), gly-his-lys (10 ng/mL), Sigma, somatostatin (10 ng/mL; Sigma), and TSH (5 milliunits/mL; Sigma) and allowed to spread and reach confluence before being replated for the proliferation assays. RAD001 (20 μmol/L stock solution in DMSO) was dissolved in culture medium and added to the cells 24 h after plating. At the indicated time points, cells were either subjected to...
WST-1 assay (see below) or harvested in radioimmunoprecipitation assay buffer for protein analysis and in Trizol (Invitrogen) for RNA analysis.

**Immunohistochemical analysis.** The following antibodies were used: rabbit polyclonal against Ki-67 (Vector Laboratories) and rabbit monoclonal (immunohistochemical specific) against pS6 (Ser235/236; Cell Signaling). Tissues were fixed, embedded in paraffin, and sectioned at 6 μm. Sections were subjected to antigen retrieval in 0.1 mol/L sodium citrate and counterstained with hematoxylin.

**Proliferation analysis.** Ki-67–stained thyroid sections were photographed at ×400 magnification and analyzed using the ImageJ software. Between 1,500 and 3,000 cells per slide were analyzed. FTC-133 and primary thyrocyte proliferation was measured in 96-well plates by WST-1 assay (Roche) according to the manufacturer’s instructions and by trypan blue exclusion from 6-well plates cultures. Primary thyrocytes proliferation was also assayed measuring BrdUrd incorporation >24 h in cells grown in chamber slides, using the BrdUrd Labeling and Detection kit II (Roche).

**Terminal deoxynucleotidyl transferase–mediated dUTP-biotin end labeling assay.** Five-micrometer-thick paraffin sections were used for programmed cell death analysis. Sections were stained using the ApopTag peroxidase *in situ* apoptosis detection kit (Chemicon International) according to the manufacturer’s instructions. Briefly, after deparaffinization and rehydration, the section were treated with Proteinase K (20 μg/mL) for 15 min. Endogenous peroxidase activity was quenched in 3.0% hydrogen peroxide in PBS for 10 min at room temperature. Sections were then treated with equilibration buffer and the working strength terminal deoxynucleotidyl transferase enzyme was applied on sections at 37°C for 60 min. Next,
the sections were incubated with antidioxygenin peroxidase conjugate for 30 min and were developed with 3,3'-diaminobenzidine tetrahydrochloride. The sections were then washed, counterstained with hematoxylin, dehydrated with alcohol, cleared in xylene, and mounted. In each staining experiment, known terminal deoxynucleotidyl transferase–mediated dUTP-biotin end labeling (TUNEL)-positive samples (cavitating embryoid bodies) were used as positive controls.

**Western blot.** Thyroids were washed with PBS and homogenized on ice in cell extraction buffer (Invitrogen) supplemented with Complete proteinase inhibitor tablet (Roche). After centrifugation and protein determination, lysates (20–40 μg) were analyzed by SDS-PAGE. Western blot analysis was carried out with the following antibodies from Cell Signaling: AKT, pAKT (Ser473), S6K1, pS6K1 (Thr421/Ser424), pS6 (Ser235/236), tuberous sclerosis (TSC)2, and pTSC2 (Thr1462); and with antibodies against cyclins D1 and D3 (Becton Dickinson) and p27Kip1 (Lab Vision).

**Reverse transcription-PCR.** Total RNA was extracted with TRIzol from pooled glands. Two micrograms of RNA were reverse transcribed using the Thermoscript kit (Invitrogen). cDNAs were diluted 10-fold and 5 μL were used for reverse transcription-PCR (RT-PCR). p27Kip1, cyclin D1, and cyclin D3 were amplified for 28 cycles, whereas 18S RNA was amplified for 15 cycles, to ensure that the analysis was carried out in the linear amplification phase. Primer sequences are available upon request.

**Statistical analysis.** All experiments were performed at least thrice. Data are expressed as mean ± SD and analyzed using *t* test.

**Results**

Chronic PI3K activation, achieved through the tissue-specific deletion of the *Pten* gene, results in increased proliferation of the thyroid follicular cells, which in turn leads to thyroid hyperplasia and adenoma (18). To assess the effect of mTOR activation in this process, we treated cohorts of 4-week-old control and *PtenLlt-TPO-Cre* mice (*n* = 7–10) with a specific mTOR inhibitor, RAD001 (everolimus). At age 4 weeks, the mutant thyroids are already homogeneously hyperplastic because *Pten* deletion has occurred around 14.5 to 16.5 dpc. (18).

At the end of a 2-week RAD001 treatment, the histology of thyroids from treated mice did not markedly differ from that of untreated glands, irrespective of their genotype (Fig. 1A and B). However, the phosphorylation level of ribosomal protein S6, a well-established mTOR downstream target, was substantially decreased by the pharmacologic inhibition of mTOR signaling (Fig. 1C and D), proving that the RAD001 regimen had effectively reached its target. These data suggest that mTOR inhibition (at least at this RAD001 concentration and for this treatment duration) cannot completely revert the hyperplastic features of an already enlarged gland.

Nevertheless, mTOR inhibition slowed down the growth of mutant thyroids, resulting in a significant and reproducible reduction (about 30%) of the thyroid weight compared with that of age-matched untreated mutants, whereas no significant effect of RAD001 was evident in wild-type mice (Fig. 2A). More strikingly, RAD001 treatment of *Pten* mutant mice restored almost wild-type proliferation levels, as measured by Ki-67 staining, suggesting that the proliferative advantage of *Pten* mutant cells heavily relies on the activity of the mTOR kinase (Fig. 2B). In contrast, the extremely low proliferation index of wild-type thyroids was not significantly altered by mTOR inhibition.

Finally, RAD001 did not induce an apoptotic response in the thyroids of control or mutant mice (Fig. 2C), indicating that mTOR inhibition only affects thyrocyte proliferation. Thus, the thyroid weight reduction observed in treated mutant is not due to cell loss but rather to a reduced growth rate in the presence of RAD001.

To define in detail the molecular mechanisms involved in mTOR-mediated thyroid cell proliferation, we first analyzed by Western blotting the expression level and activation status of relevant targets of the PI3K/AKT mitogenic cascade in the thyroids of control and mutant mice, treated or not with RAD001. As expected, AKT was found to be phosphorylated on Ser473 and, thus, activated upon loss of *Pten* (and PI3K activation) in mutant thyrocytes (Fig. 3A). As a consequence, its direct target TSC2 was ...
phosphorylated on Thr\textsuperscript{1462} in mutant cells. Notably, both AKT and TSC2 phosphorylation levels did not change upon mTOR inhibition.

AKT-dependent phosphorylation and inhibition of TSC1/TSC2 complexes and the subsequent activation of Rheb allow the activation of the mTOR kinase (20). In fact, mTOR downstream targets, S6K1 and ribosomal protein S6 (21), were found to be phosphorylated upon Pten loss, and their activation status returned to undetectable levels upon RAD001 treatment (Fig. 3A). Phosphorylation of the other major mTOR target, 4E-BP1, was not detectable in thyroid extracts under any of the treatment conditions (data not shown). Taken together, these data suggest that Pten loss and AKT activation in the thyroid follicular cells result in the constitutive activation of the mTOR pathway, which can be pharmacologically inhibited by RAD001 treatment.

mTOR activation can increase the proliferation rate through the increased translation of cell cycle effector such as cyclin D1 (22) and cyclin D3 (23). In addition, RAD001 might control the protein levels of p27Kip1 by down-regulating its specific ubiquitin ligase subunit, Skp2 (24). We found both cyclin D1 and cyclin D3 proteins up-regulated in mutant thyroids compared with wild-type controls, whereas p27Kip1 levels were not altered by Pten loss (Fig. 3A). RAD001 treatment decreased cyclin D1 to a certain extent and, more drastically, cyclin D3 expression levels both in mutant and in control thyroids, and did not have any effect on the protein levels of p27Kip1.

Strikingly, semiquantitative RT-PCR showed no difference in cyclin D1, cyclin D3, and p27Kip1 mRNA levels between untreated control, untreated mutant, and RAD001-treated thyroids (Fig. 3B), indicating that at least upon chronic PI3K activation, mTOR does not affect the transcription rate of these genes.

The dramatic effect of mTOR inhibition on cell proliferation observed in vivo was further confirmed using primary thyrocyte cultures from wild-type and mutant mice. Even a low dose RAD001 treatment (1 nmol/L) caused, at 72 h, a 50% reduction of wild-type thyrocyte proliferation compared with untreated controls and, more strikingly, resulted in a near to 70% reduction of the mutant cells growth (Fig. 4A). BrdUrd incorporation analysis in Pten\textsuperscript{-/-} thyrocytes showed that RAD001 treatment inhibits DNA synthesis (Fig. 4B).

To validate our findings in an additional more genetically complex system, we used a PTEN null thyroid follicular cancer cell line, FTC-133. Cells were treated with increasing doses of RAD001, and proliferation was assessed 72 h later. mTOR inhibition resulted in a drastic reduction of cell proliferation even at low RAD001 doses (1 nmol/L; Fig. 5A), strongly suggesting that mTOR activity is crucial also for FTC-133 proliferation. Next, we followed the growth of FTC-133 cells over the course of 3 days in the presence of 50 nmol/L RAD001 and found that mTOR inhibition progressively reduces cell proliferation (Fig. 5B) without affecting cell survival (data not shown).

In agreement with the in vivo data, RAD001 treatment reduced cyclin D1 and, more robustly, cyclin D3 protein levels, thus confirming that mTOR activation contributes to cell proliferation at least in part by increasing the levels of these two proteins (Fig. 5C). Finally, similar to what we had observed in our mouse model, the RNA levels of both cyclin D1 and cyclin D3 was not significantly altered by mTOR inhibition, strongly suggesting that up-regulation of these two cyclins takes place primarily at the translational level.

**Discussion**

Accumulating clinicopathologic as well as molecular evidence strongly suggest that alterations in various nodes of the PI3K/AKT/PTEN pathway play an extensive, previously unrecognized role in thyroid tumorigenesis (9, 10, 18, 25–28). In turn, many different downstream effectors can contribute, to different extents, to the hyperproliferative phenotype consequent PI3K activation (29). Among these, mTOR is of particular interest due to its growing involvement in the control of cell proliferation and its relevance as a pharmacologic target (30–32).

Our findings strongly support a model in which mTOR functions as a key effector of PI3K-generated proliferative signals by increasing the protein levels of cyclins D1 and D3 through posttranscriptional mechanisms, most likely increased translation. Furthermore, they show that RAD001-mediated mTOR inhibition can effectively restore normal D-type cyclin protein levels and, thus, normal proliferation rates in thyroid follicular cells in which the PI3K/AKT/mTOR axis is constitutively activated.

We have recently shown that, surprisingly, also the in vivo proliferative response to chronic thyroid-stimulating hormone receptor (TSHR) stimulation heavily relies on the activation of the mTOR/S6K1 axis, and that mTOR inhibition during goitrogenic stimulation abrogates the hyperplastic thyrocyte responses to

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**Figure 4.** mTOR inhibition reduces cell proliferation in primary mouse thyrocytes. A, dose-response curve of wild-type and mutant thyrocytes after 72 h of RAD001 treatment, analyzed by WST assay. Results are shown as ratio to untreated cells. *, P = 0.001 (t test). B, BrdUrd incorporation >24 h in mutant cells grown in the absence or presence of 10 nmol/L RAD001. *, P = 0.01 (t test).
References

7. BT-474 cells and cyclin D2 expression in FTC-133 cells after 24 and 48 h RAD001 treatment (50 nmol/L). D, RT-PCR analysis of the effect of RAD001 treatment on the expression of cyclin D1 and cyclin D3 in FTC-133 cells after 24 and 48 h RAD001 treatment (50 nmol/L).

Figure 5. mTOR inhibition reduces cell proliferation in thyroid cancer cells. A, dose-response curve of FTC-133 cells after 72 h of RAD001 treatment, analyzed by WST assay. *, P = 9.8 x 10^(-5) (t test). B, time course of cell growth after 50 nmol/L RAD001 treatment. **, P = 0.01; ***, P = 0.001 (t test). C, Western blot analysis of S6K1 phosphorylation and cyclins D1 and D3 expression in FTC-133 cells after 24 and 48 h RAD001 treatment (50 nmol/L).
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