Lean Body Weight and Metformin Are Insufficient to Prevent Endometrial Hyperplasia in Mice Harboring Inactivating Mutations in PTEN

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Key Words

Endometrial hyperplasia · Metformin · Mouse models · Obesity · Prevention · Phosphatase and tensin homolog

Abstract

Objectives: Obesity is a major risk factor for endometrial cancer. We evaluated whether obesity exacerbates progression of endometrial hyperplasia (EH) using the PR^{Cre/+} PTEN^{flox/+} mouse model and examined if the type 2 diabetes drug, metformin, could prevent EH. **Methods:** Twenty obese (PR^{Cre/+} PTEN^{flox/+}) mice were maintained on a high-fat diet, while 20 lean mice ate a matching low-fat diet. Ten mice from each group received metformin (1,000 mg/day) in drinking water. Mice were euthanized at 26 weeks. Uterine tissue was scored for degree of EH. Immunohistochemical staining for Ki67 was used to evaluate cellular proliferation. Markers of PI3K/AKT/mTOR activity were evaluated by immunohistochemistry using activation-specific antibodies. Serum adiponectin was quantified by ELISA. **Results:** Obesity had no effect on the extent of EH in (PR^{Cre/+} PTEN^{flox/+}) mice. While metformin significantly altered circulating adiponectin levels in obese and lean animals, it had no effect on EH. There were no differences in endometrial proliferation as measured by Ki67 staining. Neither obesity nor metformin altered PI3K/AKT/

mTOR activity in these animals. **Conclusions:** Weight and metformin did not affect the severity of EH resulting from PTEN inactivation. Alternative mouse models of early endometrial cancer are required for preclinical cancer prevention studies.

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Introduction

Endometrial cancer (EC) is the most common gynecologic malignancy in the US, with an estimated 52,630 new cases diagnosed in 2014 [1]. Endometrial tumors have traditionally been classified into two types [2–4]. Type I or endometrioid endometrial carcinomas, which represent more than 80% of all EC cases, are associated with obesity, hyperinsulinemia, and prolonged exposure to unopposed estrogen. These tumors are thought to arise from the progression of endometrial hyperplasia (EH) to complex atypical hyperplasia to well-differentiated endometrial carcinomas (EC). Type II or nonendometrioid endometrial carcinomas, include uterine serous carcinomas, and are typically more aggressive and estrogen independent. Recently, based on extensive genomic analyses of both type I and type II tumors, ECs have been further

reclassified into multiple subtypes [2, 5–8]. Indeed, the detailed characterization of molecular alterations underlying the pathogenesis of the different histologies of EC lays the groundwork for rational disease prevention strategies and therapeutic drug design [2, 5–8].

The most frequently observed genetic mutations in type I EC occur in the phosphatase and tensin homolog (PTEN) tumor suppressor gene. PTEN mutation is thought to be an early event in the pathogenesis of EC, and its incidence increases with disease progression [9]. Up to 20% of cases of EH harbor mutations in PTEN, while over 80% of type I ECs possess inactivating mutations in PTEN [10]. PTEN acts as both a protein and lipid phosphatase [11, 12]. It preferentially dephosphorylates phosphatidylinositol-3,4,5-trisphosphate (PIP3), thereby acting in opposition to phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K). Mutations resulting in the loss of function or expression of the PTEN protein, are associated with hyperactivity of the PI3K/AKT/mTOR pathway, thereby increasing cellular proliferation and survival, metastasis and angiogenesis, changes in metabolism and resistance to chemotherapeutic agents [11–13].

Genetically modified PTEN ‘knockout’ mice represent a well-characterized animal model for the study of the progression of EH to type I EC. Without fail, all female mice heterozygous for inactivating mutations in PTEN develop EH by 26 weeks of age, with 20% of mice progressing to low grade EC by 40 weeks [14–16]. This effect is observed in mouse models with systemic heterozygous disruption of PTEN (PTEN^{+/-}) and in models targeting heterozygous disruption to the endometrium using a Cre-Lox system (PR^{Cre/+} PTEN^{flox/+}). We previously demonstrated that everolimus (RAD001), an mTOR inhibitor, prevented the progression of EH in these animals [17], indicating that this animal model might be useful to study the chemoprevention of EH.

Obesity greatly increases the risk for EC [3]. The American Institute for Cancer Research estimates that 59% of ECs can be prevented by maintaining a healthy weight and by being physically active [18]. PR^{Cre/+} PTEN^{flox/+} mice bred on a C57BL/6 background are susceptible to diet-induced obesity, with accompanying mild to moderate hyperglycemia and hyperinsulinemia, which mimic the human condition [19–21]. Given the role of obesity in type-1 EC risk, we sought to determine if obesity would accelerate the onset of EH and its progression to EC in the PR^{Cre/+} PTEN^{flox/+} mouse model.

Metformin, a biguanide drug commonly used to treat type 2 diabetes, has recently been shown to exert growth-

inhibitory effects for a variety of cancers, including EC both in vitro and in vivo [22–25]. Recent studies by our own group have demonstrated endometrial responsiveness to metformin therapy in a rat model [24, 26]. Metformin decreases cellular proliferation by multiple mechanisms. Metformin increases insulin receptor sensitivity and decreases circulating insulin levels, thereby modulating insulin receptor-mediated growth. Metformin also activates the growth-inhibitory AMP-dependent kinase (AMPK) pathway, which counteracts signaling through both the PI3K/AKT and MAPK pathways downstream of a variety of growth factor receptors [22, 27]. Therefore, in addition to examining the effects of obesity on the pathogenesis of EH, we further investigated whether metformin could act as a chemopreventive agent in PR^{Cre/+} PTEN^{flox/+} mice whether lean or obese.

Materials and Methods

Animals

All procedures described were performed following approval by the MD Anderson Cancer Center Institutional Animal Care and Use Committee, and in adherence to the institutional guidelines. PR^{Cre/+} PTEN^{flox/flox} mice were obtained from Dr. F. Demayo, Baylor College of Medicine [28, 29]. Cre recombinase is expressed under the regulation of the progesterone receptor promoter [28]; therefore, in a floxed PTEN female mouse, PTEN is selectively disrupted in PR-expressing tissues, including the endometrium, oviduct, ovary, mammary gland, and pituitary [28]. Male PR^{Cre/+} PTEN^{flox/flox} which demonstrate no obvious phenotype, were crossed with wild-type C57BL/6 females to generate PR^{Cre/+} PTEN^{flox/+} mice. At 6 weeks of age, female mice were divided into two treatment groups (n = 20/group). Twenty mice were fed a high-fat diet (HFD) in which 60% of calories were obtained from fat (D12492 Research Diets Inc., New Brunswick, NJ) while 20 mice were fed a matching, low-fat diet (LFD; D12450B). Mice were weighed weekly to monitor weight gain. At 10 weeks, when all HFD animals had reached a weight 20% greater than that of LFD controls, mice were again divided into two treatment groups (n = 10). Ten HFD and 10 LFD mice received metformin (5 mg/ml) in their drinking water, while controls received untreated water. Animals continued with their respective diets. Both groups drank the same volume of liquid daily, with metformin-treated mice receiving a dose of approximately 1,000 mg/kg daily. All animals were euthanized at 26 weeks of age, when 100% of lean, untreated controls were expected to develop EH (control group). Serum and uterine tissue were collected for further evaluation.

Scoring for EH

Murine endometrial tissues were evaluated for EH as previously described [17]. Briefly, hematoxylin and eosin-stained sections of endometrium were reviewed, to determine hyperplasia and grade by a gynecologic pathologist (R.R.B.) who was blinded

Table 1. The effects of obesity and metformin on degree of endometrial hyperplasia and proliferative signaling in obese and lean PR^{Cre/+} PTEN^{fl/fl} mice

	LFD control			LFD + metformin				HFD				HFD + metformin			
	n	mean	SD	n	mean	SD	p	n	mean	SD	p	n	mean	SD	p
Hyperplasia, score	10	4.70	1.89	10	5.90	2.47	0.54	10	5.90	2.64	0.54	10	6.10	2.38	0.42
Tissue biomarkers															
Ki67, % positive	10	21.2	14.6	10	29.3	18.9	0.67	10	30.5	20.3	0.57	10	29.0	21.8	0.69
pS6rp, IHC score	10	5.40	2.12	10	5.00	2.11	0.98	10	5.40	3.50	1.0	10	4.30	3.16	0.71
pACC, IHC score	10	2.40	0.52	10	2.40	0.52	1.0	10	2.30	0.48	0.95	10	2.50	0.53	0.95
Caspase 3, IHC score	10	4.70	7.85	10	4.75	5.28	1.0	10	6.65	6.46	0.88	10	7.65	8.76	0.69
pERK, IHC score	10	7.00	2.58	10	8.00	3.50	0.75	10	7.00	2.11	1.0	10	6.00	2.73	0.75
pAKT, IHC score	10	1.60	0.52	10	1.80	0.42	0.78	10	1.80	0.79	0.78	10	1.50	0.53	0.96
Serum biomarkers															
Adiponectin, ng/ml	10	14.5	2.41	9	10.5	1.96	0.002	8	15.4	3.22	0.78	8	10.9	1.40	0.007 0.03 ^a

Unless otherwise noted, all comparisons are made with the LFD control animals. ^a Adiponectin levels were significantly reduced in the HFD + metformin group when compared to HFD alone, thereby confirming a systemic metformin response in obese animals.

to the study groups. The description of the hyperplastic lesions in the PTEN heterozygote has not been consistent in previous reports [14, 15, 30, 31]. In contrast to human EH that progresses from simple hyperplasia without atypia to complex hyperplasia with atypia, mouse EH, even in very early hyperplasia, displays cytologic atypia. Therefore, to have a more consistent and quantitative description, we defined 3 grades of hyperplasia according to the percentage and size of the hyperplastic lesions. A score describing the extent of hyperplasia was as follows: 1 = <33% hyperplasia; 2 = 33–66% hyperplasia; 3 = >66% hyperplasia. Severity of hyperplastic lesions was scored as follows: (1) the hyperplastic glands were small and regular; (2) mix of small and large size; (3) more than one half of the hyperplastic glands were large and irregular. The overall score for each case was defined by the product of the extent of hyperplasia score and score for severity of the lesion. The hyperplastic lesion was defined as grade 1 when the product of the 2 scores was 1–3, as grade 2 when the product was 4–6, and as grade 3 when the product was 7–9.

Immunohistochemical Staining of Endometrial Tissues

The effect of obesity and metformin on mouse endometrial proliferation was evaluated in formalin-fixed, paraffin embedded tissue by Ki67 staining (BD Biosciences, San Diego, CA, USA) following the manufacturer's instructions. The degree of apoptosis in endometrial tissues was determined by immunohistochemistry (IHC) staining for cleaved caspase-3 (Asp175; Cell Signaling, Danvers, MA, USA). The sections were counterstained with Mayer's hematoxylin. The average number of positively-stained Ki67 or caspase-3 cells in 5–10 high-power microscopic fields were counted per slide and calculated as: 200× (number of positively stained endometrial cells/total endometrial cells). Tissues were also stained for downstream markers of insulin and IGF-1 receptor signaling, including phospho-S6 ribosomal protein (Ser235/236), phospho-AKT (Ser473), phospho-p44/42 MAPK (Thr202/Tyr204), and phospho-acetyl-CoA carboxylase (Ser79) (Cell Signaling, Danvers, MA, USA) and scored according to previously published procedures [24].

Serum Analyses

Serum adiponectin levels were evaluated using the Mouse Adiponectin ELISA Kit (Millipore, Billerica, MA, USA). Serum samples were tested in triplicate.

Statistical Analysis

For each of the following tissue and serum markers: Ki67, pS6rp (phospho-S6 ribosomal protein), pACC (phospho-acetyl-CoA carboxylase), caspase 3, pERK (phospho-p44/42 ERK), pAKT (phospho-AKT), and adiponectin, a one-way analysis of variance was calculated. Dunnett's test was used to conduct post hoc comparisons of the LFD group (control) with the experimental groups (diet and metformin). All tests were two-sided, and $p < 0.05$ was considered to be statistically significant.

Results

LFD and Metformin Are Insufficient to Decrease the Extent of EH in PR^{Cre/+} PTEN^{fllox/+} Mice

Histological scores between treatment groups and the control group were not statistically different (fig. 1; table 1). Mean hyperplasia scores were 4.7, 5.9, 5.9, and 6.1, for control lean mice, obese mice, and metformin-treated lean and obese mice, respectively, with the majority of mice presenting with grade 2 hyperplasia as defined by our scoring system at 26 weeks, irrespective of treatment group. The effect of metformin and diet/obesity on endometrial proliferation was compared using IHC staining for Ki67. Despite a significant weight difference in the HFD versus LFD groups (fig. 1a), no significant differences in EH were observed at 26 weeks (fig. 1b). Further-

Fig. 1. Obesity and metformin treatment have no detectable effect on the extent of EH in the $PR^{Cre/+}$ $PTEN^{fllox/+}$ mouse model. **a** $PR^{Cre/+}$ $PTEN^{fllox/+}$ mice were fed either an HFD or LFD and either untreated or treated with metformin (1,000 mg/kg) in drinking water. Animals were weighed and euthanized at 26 weeks. Serum and reproductive organs were collected for analysis. LFD-fed mouse weights, either treated or untreated with metformin, were significantly lower than those in HFD-fed mice. There were no

significant changes in weight in response to metformin in either HFD- or LFD-fed animals. **b** Endometrial tissue was evaluated for the presence of hyperplasia following HE staining, and cellular proliferation was analyzed by IHC staining for Ki67. **c** No significant difference in cell proliferation was observed between treatment groups, as determined by percentage of Ki67-positive cells in the endometrium.

more, metformin produced no significant changes in animal weight or on the development of EH in either obese or lean animals. Taken together, no significant difference in hyperplasia score or endometrial proliferation was observed between any of the four treatment groups (fig. 1; table 1).

Markers of Cellular Proliferation and Insulin/IGF-1 Signaling Are Unaffected by either Diet-Induced Obesity or Metformin in the Endometrium of $PR^{Cre/+}$ $PTEN^{fllox/+}$ Mice

Changes in signaling pathways typically affected by obesity and metformin were evaluated in the endome-

trium by IHC staining using activation-specific antibodies. Findings are summarized in table 1. No significant differences in IHC staining scores for pS6rp (Ser235/236), pAKT (Ser473), pERK (Thr202/Tyr204), and pACC (Ser79) were observed between the endometrial tissues of any of the three treatment groups as compared to the LFD control group. Furthermore, no differences in apoptosis, as measured by caspase 3 IHC staining, were observed between control lean mice, obese mice, and metformin-treated lean and obese mice.

Metformin Suppresses Adiponectin Expression in Both Obese and Lean PR^{Cre/+} PTEN^{fllox/+} Mice

Recent studies by our group have demonstrated endometrial responsiveness to metformin therapy in a rat model [24]. The apparent lack of effect on signal transduction in the endometrium of PR^{Cre/+} PTEN^{fllox/+} mice led us to examine whether this animal model was systemically resistant to the effects of metformin.

Insulin levels could not be used as a marker for metformin response as animals in this study were not fasted; however, Huypens et al. [32] observed that metformin reduces the expression of adiponectin expression and release in mouse (3T3-L1) adipocytes. We therefore tested the serum levels of adiponectin as a surrogate marker of response to metformin (table 1). As compared to control lean mice (14.5 ng/ml), adiponectin levels decreased significantly with metformin treatment in both lean (10.5 ng/ml, $p = 0.002$) and obese (10.9 ng/ml, $p = 0.007$) mice. These findings confirm that PR^{Cre/+} PTEN^{fllox/+} mice do have a systemic response to metformin, despite the apparent lack of response to this drug in endometrial tissue.

Discussion

Inactivating mutations in the PTEN tumor suppressor gene, are commonly observed in type I EC, and are thought to represent an early event in the pathogenesis of this disease. In support of this hypothesis, 100% of female mice heterozygous for inactivating mutations in PTEN develop EH by 26 weeks of age, with 20% of these mice progressing to grade 1 EC by 40 weeks of age. Our previous studies have demonstrated that PTEN heterozygous mice represent a useful model for the preclinical evaluation of chemotherapeutic drugs, such as everolimus, which may prevent the progression from EH to EC. The findings presented in the current study, however, suggest that this model may be less useful in the evaluation of behavioral therapies, such as diet and exercise, or drugs

such as metformin, which are intended to prevent or reverse the earliest transformation from normal to hyperplastic endometrium.

In the PR^{Cre/+} PTEN^{fllox/+} mouse model, diet-induced obesity did not increase the degree of EH as compared to lean animals fed an LFD. There was no significant difference in Ki67 staining, which is a tissue marker of proliferation. Signaling through the PI3K/AKT and MAPK pathways was unaffected by obesity in endometrial tissue possessing a genetic mutation of PTEN. Furthermore, despite influencing systemic levels of adiponectin, the anti-diabetic drug metformin had no apparent effect on EH in either obese or lean PR^{Cre/+} PTEN^{fllox/+} mice. Taken together, these data suggest that a genetically acquired PTEN mutation in the endometrium was sufficient to drive the development of EH, and that these lesions are resistant to the effects of obesity and metformin.

We have previously demonstrated that everolimus (RAD001) inhibits the progression of EH in PTEN knockout mice. PTEN is a physiological inhibitor of PI3K/AKT/mTOR pathway. Everolimus (RAD001) specifically inhibits mTOR kinase activity, and inhibits proliferation, even in the absence of PTEN. Our current studies suggest that systemic changes in proliferative signaling in response to diet/exercise/metformin, are insufficient to inhibit PI3K/AKT/mTOR signaling and cannot overcome the progression of EH driven by germline mutations in PTEN. It is important to note that Fyles et al. [33] have shown that progestin treatment does not prevent endometrial neoplasia in PTEN^{+/-} mice. The lack of hormone effect is also seen in ovariectomized animals, suggesting that progression of PTEN^{+/-}-associated hyperplasia is not driven by estrogen. Joshi et al. [34] have confirmed these findings and demonstrated that endometrial tumorigenesis occurs independently of estrogen or estrogen receptors in the PTEN^{+/-} mouse model. And while Kim et al. [35] demonstrated a decrease in overall uterus and tumor size in progesterone-treated, ovariectomized PR^{Cre/+} PTEN^{flf} (homozygous loss of PTEN) animals, progesterone ultimately did not prevent cancer in these animals. It is essential to consider these observations in determining the use of this model for translational research questions, as up to 80% of early EH are responsive to hormonal therapy in humans [36].

We conclude that, while the PTEN heterozygous knockout mouse model remains useful for the preclinical evaluation of interventions for Cowden disease-associated EC and for treatment of sporadic EC driven by PTEN mutations, it is not suitable for all studies directed at the chemoprevention of early EC.

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Disclosure Statement

The authors report no conflict of interest.

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