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Genistein promotes DNA demethylation of the steroidogenic factor 1 (SF-1) promoter in endometrial stromal cells

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ABSTRACT

It has recently been demonstrated that genistein (GEN), a phytoestrogen in soy products, is an epigenetic modulator in various types of cells; but its effect on endometrium has not yet been determined. We investigated the effects of GEN on mouse uterine cells, *in vivo* and *in vitro*. Oral administration of GEN for 1 week induced mild proliferation of the endometrium in ovariectomized (OVX) mice, which was accompanied by the induction of steroidogenic factor 1 (SF-1) gene expression. GEN administration induced demethylation of multiple CpG sites in the SF-1 promoter; these sites are extensively methylated and thus silenced in normal endometrium. The GEN-mediated promoter demethylation occurred predominantly on the luminal side, as opposed to myometrium side, indicating that the epigenetic change was mainly shown in regenerated cells. Primary cultures of endometrial stromal cell colonies were screened for GEN-mediated alterations of DNA methylation by a high-resolution melting (HRM) method. One out of 20 colony-forming cell clones showed GEN-induced demethylation of SF-1. This clone exhibited a high proliferation capacity with continuous colony formation activity through multiple serial clonings. We propose that only a portion of endometrial cells are capable of receiving epigenetic modulation by GEN.

1. Introduction

Genistein (GEN), a major phytoestrogen in dietary soy, is a substantial component of the typical Asian and Western vegetarian diets, as well as recently developed infant soy milk formulas. There are several well known potential health benefits of GEN intake [1,2], one of which is an apparent decreased risk of breast and prostate cancers, based on human observational studies [1,3]. But GEN also paradoxically stimulates growth of breast cancer cells in culture [2] and uterine enlargement in rodents [4]. These effects may be mediated through estrogen receptor interactions and/or modulation of endogenous estrogen metabolism [5,6]. Since GEN can bind to estrogen receptors (ERs) α and β, with a stronger affinity to ERβ [5], it is categorized as a phyto-selective estrogen receptor modulator (SERM) [6,7]. The variations in GEN's agonistic or antagonistic effects may be affected by variations in endogenous estrogen levels. Previous studies have not determined whether the pleiotropic effects of GEN involve distinct epigenetic alteration. Recently, GEN was shown to alter DNA methylation in various types of cells, including ES cells [8], but most studies have been performed using cancer cell lines [9–11]. There have been few reports of the effects of GEN on DNA methylation in intact cells or *in vivo* [12]. In the present study, we utilized a uterotropic assay in ovariectomized (OVX) mice, as a model system to analyze epigenetic regulation by GEN.

In a previous study, high-dose GEN administration to OVX rats resulted in increased uterine weight and changed endometrial cell gene expression [6]. However, no epigenetic alterations were demonstrated under this condition. We selected the steroidogenic factor 1 (SF-1; official symbol: Nr5a1) gene as a target for the methylation analysis. SF-1 is an orphan nuclear receptor and transcription factor for key enzymes involved in steroidogenesis, such as StAR, Cyp11a1 (p450scc), Cyp17a1 (p450c17), and Cyp19a1 (aromatase) [13]. The SF-1 gene is not expressed in normal endometrium; however, SF-1 expression is reactivated in the disease state of human ectopic endometriosis, in which the SF-1 promoter is abnormally demethylated by an unknown mechanism [14]. The subsequent enhancement of steroidogenic genes and resultant local steroidogenesis are proposed to be important etiologies [15]. Therefore, we hypothesized that in mouse endometrial cells,

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SF-1 might be subjected to epigenetic modulation by some external stimuli. Here we show that the SF-1 promoter was demethylated *in vivo* and *in vitro* by GEN treatment. This is the first demonstration of a phytoestrogen altering the epigenetic state of adult endometrium.

2. Materials and methods

2.1. Ethics statement

All procedures described here were performed according to protocols approved by the Animal Care Committee of the National Institute of Health Sciences, and Tokyo Medical and Dental University (No. 0110306A).

2.2. Oral administration of genistein to ovariectomized mice

C57BL/6JJmsSlc female mice (SLC) were used in this study. All mice were fed a phytoestrogen-free diet (Oriental Yeast) and were ovariectomized (OVX) 2 weeks prior to the genistein (GEN) treatment. OVX mice were divided into three different treatment groups, each consisting of 3–5 independent replicates, which orally received low-dose GEN (60 mg/kg/day), high-dose GEN (200 mg/kg/day), or vehicle (0.5% CMC-Na (Maruishi Pharmaceutical); 5 ml/kg/day) for 1 week. At the end of treatment (9 weeks of age), all mice were euthanized by exsanguination under ether anesthesia.

2.3. Uterotrophic assay and gene expression study after oral administration of genistein

Whole uteri were harvested, blotted, and weighted. Each uterus was divided into two horns, immediately placed into 2 ml plastic tubes of RNAlater solution (Ambion), and stored at 4 °C. From each sample, one horn was processed for mRNA expression analyses; RNAlater was replaced with 1.0 ml of RLT buffer (Qiagen), and the horn was homogenized by addition of a 5 mm diameter Zirconium bead (Funakoshi) and shaking with a MixerMill 300 (Qiagen) at 20 Hz for 5 min (only the outermost row of the shaker box was used). Further sample preparation and analysis were performed as previously described [16]. mRNA expressions were analyzed using Affymetrix Murine Genome 430 2.0 GeneChips, and calculated as copy number per cell by the Percellome method [16]. The second uterine horn of each sample was subjected to genomic DNA isolation.

2.4. Isolation of colony-forming cells derived from intact uteri

Five 8- to 9-week-old C57BL/6JJmsSlc female mice (SLC) were euthanized by cervical dislocation and whole uteri were harvested. Uterine horns were collected in Dulbecco's modified Eagle's medium/Hams F-12 (DMEM/F-12; Nacalai Tesque) containing 0.05 mg/ml gentamicin (Sigma–Aldrich). Each horn was dissected longitudinally and the endometrial tissue was divided into two portions: the luminal side and the myometrium side. A single cell suspension of endometrial cells was obtained using enzymatic digestion and mechanical means adapted from Chan et al. [17]. The tissue samples were minced and dissociated in 500 μl DMEM/F-12 containing 0.12 mg/ml (0.56 Wünsch U/ml) Blendzyme 2 and 40 μg/ml deoxyribonuclease type I (both from Roche Applied Science) in a shaking incubator (~90 rpm) at 37 °C. At 15 min intervals, the digests were pipetted to promote separation and cell dissociation was monitored microscopically. After 45 min, debris was filtered out using a 40-μm sieve (BD Biosciences). The single-cell suspensions were collected in DMEM/F-12 containing 10% FBS, 0.05 mg/ml gentamicin and stored on ice. Then the sieves were backwashed, and myometrial and glandular debris were further digested to single cells for 45 min as described above. All cell suspensions were filtered as described above, and combined. To remove erythrocytes, the cells were resuspended in 500 μl of HLB solution (Immuno Biological Laboratories) and incubated for 3 min. After washing twice with PBS, viable cell numbers were counted with trypan blue (Sigma–Aldrich). Cells were seeded on gelatin (0.1%, Sigma–Aldrich)-coated dishes at various densities of $0.1–3 \times 10^5$ cell/60-mm dish. After 14 days, non-overlapping clones were distinguished. Primary cell clones were expanded in DMEM/F-12 containing 5% FBS (SAFC Biosciences) and 0.05–0.1 mg/ml gentamicin, on gelatin-coated dishes.

2.5. Serial cloning of colony-forming cell clones

Self renewal was assessed by serial cloning of individual clones as described by Gargett et al. [18]. Cells were seeded on gelatin-coated 100-mm dishes at 10 cells/cm^2 (600 cells/100-mm dish). Culture medium was changed every 4 days and secondary clones formed distinct colonies by 14 days after plating. Secondary clones were similarly recloned to generate tertiary clones, and were also expanded in the same manner as the primary culture.

2.6. In vitro genistein exposure to colony-forming cells

From 70 isolated cell clones, we selected 20 clones from colonies that were composed of fibroblastic-shaped, homogenous cells with an average doubling time of less than 100 h. The selected clonal cells, whose passage number was less than 10, were subjected to *in vitro* GEN exposure. Cells were seeded on gelatin-coated 60-mm dishes, treated with or without 10 μM of GEN (dissolved in dimethylsulfoxide (DMSO)) in DMEM/F-12 containing 5% FBS and 0.05 mg/ml gentamicin for 7 days. The final DMSO concentration was 0.02%. The culture medium was changed every 2 days.

2.7. Genomic DNA preparation and bisulfite sequencing

Genomic DNA was isolated using a QIAamp DNA Mini Kit (QIAGEN) and 180 ng–1 μg was subjected to sodium bisulfite modification with a EpiTect Bisulfite Kit (QIAGEN) according to manufacturer's protocols. Bisulfite sequencing primers are shown in Supplementary Table 1. PCR products were cloned into the pT7 blue T vector (Novagen) and transformed into *Escherichia coli*. Plasmid DNA from positive colonies was purified and sequenced at the Tokyo Medical and Dental University Genome Laboratory (Tokyo, Japan). Sequence and statistical analyses were performed with the QUantification tool for Methylation Analysis; http://quma.cdb.riken.jp/top/quma_main_j.html [19]. The statistical significance of the difference between two bisulfite sequence groups at each CpG site was evaluated with Fisher's exact test.

2.8. Screening of DNA methylation status by high-resolution melting assay

All assays were performed on the LightCycler 480 using the LightCycler 480 High Resolution Melting Master kit, according to the manufacturer's instructions. Primers, designed using LightCycler Probe Design Software 2.0 (All, Roche Applied Science) are shown in Supplementary Table 1. All data were analyzed using LightCycler Gene Scanning Software.

2.9. Statistical analysis

Data are shown as means ± SD. Unpaired *t*-tests were used to compare the significance between two groups. Statistical analysis